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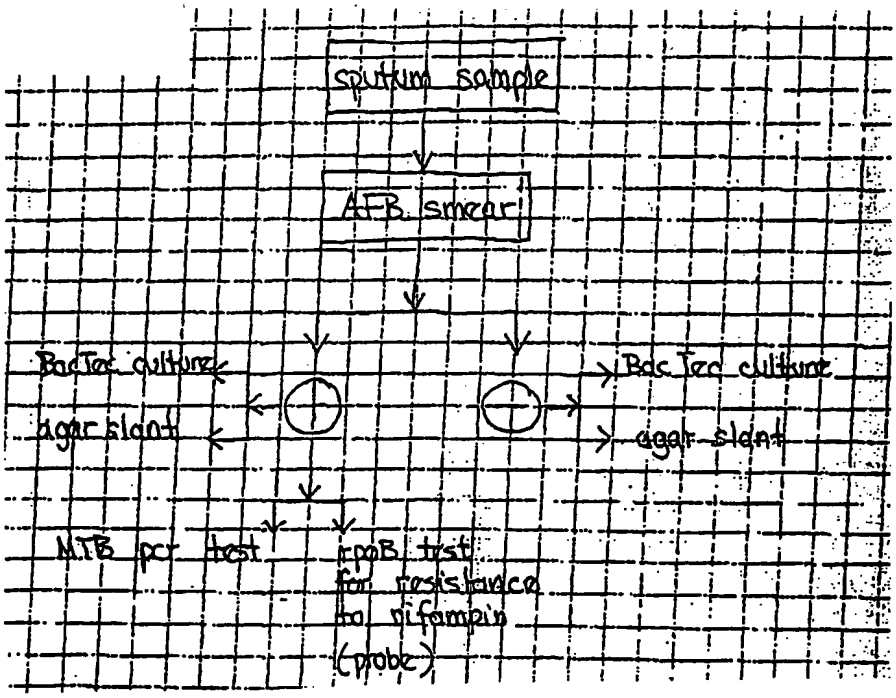
(57) Abstract

Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the rpoB (rifampin), katG (isoniazid), oxyR-ahpC PR (isoniazid), mabA (isoniazid), rpsL/s12 (streptomycin), 16S/rrs (streptomycin), embB (ethambutol), pncA (pyrazinamide), gyrA (ciprofloxacin) and 23S (azithromycin) genes of *Mycobacterium tuberculosis*. These primers can be used in a method for detection and characterization of *Mycobacterium tuberculosis* present in a sample. The method includes the steps of obtaining a sputum sample suspected of containing M. tuberculosis, performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the rpoB, katG, rpsL/s12 and 23S genes for the presence of antibiotic-resistance inducing mutations; and (c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic-resistance inducing mutations.



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METHOD AND KIT FOR THE CHARACTERIZATION OF ANTIBIOTIC-RESISTANCE MUTATIONS IN *MYCOBACTERIUM TUBERCULOSIS*

DESCRIPTION

Field of the Invention

This application relates to a method and kit for the characterization of antibiotic resistance mutations in *Mycobacterium tuberculosis*, and particularly to the evaluation of such mutations in clinical samples.

Background of the Invention

M. tuberculosis can be resistant to all antibiotics that are currently used to treat tuberculosis patients. Antibiotic resistance is due to acquired point mutations in target genes in the genome of *M. tuberculosis*. These point mutations render the organism insensitive to the action of the antibiotic by preventing it's uptake or activation, or by altering the antibiotic target. The observed antibiotic resistance in *M. tuberculosis* is not due to an episome-encoded resistance gene transferred from one strain to another and, like other bacteria, is single-step (one point mutation), high-level resistance.

Rapid and accurate detection of antibiotic resistance in *Mycobacterium tuberculosis* in sputum samples would greatly improve both patient treatment and outcome. Presently, analysis of *M. tuberculosis* is carried out on DNA recovered from sputum samples handled according to Standard Infectious Disease/Public Health Laboratory practices. The sputum sample is decontaminated and a cell sediment isolated. This cell sediment is the sample source for all routine procedures used in the detection and isolation of *M. tuberculosis*. Portions of this sample are used in BacTec cultures for selective growth of *M. tuberculosis*, agar plate/agar slant cultures for *M. tuberculosis*, acid-fast bacilli (AFB) smears for mycobacteria detection and molecular biological methods for the detection of M. tuberculosis and atypical mycobacteria. (See Fig. 1)

Mycobacterial DNA is prepared directly from the decontaminated sputum cell sediments according to standard procedures and this mycobacterial DNA is used in the various molecular biological detection procedures. The methods presently in use for the detection of *M. tuberculosis* are either PCR–based or probe-based. These tests are used

primarily on AFB smear-positive samples. Since the presence of *M. tuberculosis* has already been established by the AFB smear, these tests are used primarily in a confirmatory capacity as opposed to a diagnostic capacity. Furthermore, these tests provide no information on the potential antibiotic resistance of these *M. tuberculosis* samples.

Below is a list of antibiotics used to treat *M. Tuberculosis* infections. The gene target of the specific antibiotic and regions associated with antibiotic resistance are listed, if known. The references on which the codon assignments are based are listed at the end of the application.

1.	Rifampin	rpoB gene	codon 507-533[a]
2.	Isoniazid	katG gene	codon 275/315/328[b]
3.	Isoniazid	mabA gene	unknown[c]
4.	Isoniazid	oxyR-ahpC intergenic region (PR)	nucleotides -48 to +33
5.	Azithromycin	23S rRNA sequence	nucleotide 2568A[e]
6.	Pyrazinamide	pncA gene	codon 47/85[f]
7.	Ethambutol	embB gene	codon 306[g]
8.	Streptomycin	rpsL/s12 gene	codon 43/88[h]
9.	Streptomycin	16S/rrs sequence	nucleotides 491, 512, 516, 513, 903, 904
10.	Ciprofloxacin	gyrA gene	codon 88-95[j]

Probe-based tests do exist for the determination of rifampin resistance in *M. tuberculosis* (line probe assay-InnoTek), but these probes rely on prior knowledge of antibiotic resistance-associated mutations in the rpoB gene. Mutations outside the region covered by the probe or new mutations not included in the probe cocktail could still confer resistance, but would not be detected using this product in it's present form.

Thus, there remains a need for a method for detecting antibiotic-resistance mutations in clinical *M. tuberculosis* sputum samples which is capable of detecting mutations in all of the gene targets which confer antibiotic resistance. It is an object of the present invention to provide such a method. It is a further object of this invention to provide amplification and cycle sequencing primer sets, and kits containing such primer sets, for use in the characterization of antibiotic resistance mutations in *M. tuberculosis*.

Summary of Invention

Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the rpoB (rifampin), katG (isoniazid), oxyR-ahpC PR (isoniazid), mabA (isoniazid), rpsL/s12 (streptomycin), 16S/rrs (streptomycin), embB (ethambutol), pncA (pyrazinamide), gyrA (ciprofloxacin) and 23S (azithromycin) genes. Using these primer sets and the OPENGENE™ automated DNA sequencing system, a protocol has been developed which permits both the rapid detection of *M. tuberculosis* and the identification of antibiotic resistance–associated mutations in a series of gene targets. The present invention uses a series of tests designed to detect antibiotic resistance-associated mutation in all gene targets for all antibiotics presently in use for the treatment of tuberculosis. The tests are employed in a hierarchical manner on both AFB smear-positive or smear-negative samples to determine both the presence and antibiotic-resistance of *M. tuberculosis* in a given sample. This method permits the simultaneous determination of *M. tuberculosis* presence in a sample and the antibiotic-resistance profile to an entire panel of antibiotics. Standard methods require from 2-6 weeks to culture *M. tuberculosis* and additional time to establish antibiotic resistance. Although DNA sequence-based (genotypic) tests are not intended to replace the traditional culture-based (phenotypic) methods, these tests do represent a rapid, sensitive and accurate protocol which provides clinicians with valuable information regarding antibiotic treatment options within days as opposed to weeks.

Brief Description to the Figures

Fig. 1 shows known testing protocols for *M. tuberculosis*; and

Fig. 2 shows a hierarchical assay scheme for evaluating *M. tuberculosis* type in accordance with the invention.

Detailed Description of the Invention

In accordance with the invention, regions of the genome of *M. tuberculosis* associated with antibiotic resistance are amplified and sequenced using specifically designed amplification and sequencing primers. Various techniques for amplification are known, including the basic PCR amplification techniques described in US Patent No. 4,683,202, which is incorporated herein by reference. Similarly, various techniques for sequencing are know, some of which require prior amplification and some of which do not. Included among known sequencing techniques are those disclosed in US Patents Nos. 5,834,189 and 5,789,168, which are incorporated herein by reference. The primers of the invention can be used in any of these sequencing formats, although the invention is exemplified below using separate amplification and cycle-sequencing steps.

In theory, the selection of primers to amplify and sequence a known region of interest should be straightforward. In fact, however, because of the possibility of primer binding to other sites, complications arising from secondary structure, and other factors which are not fully understood, some primers perform better than others for amplification and sequencing of the same region of interest. The present invention provides primers which have been optimized for the amplification and sequencing of regions associated with each of the ten known types of antibiotic resistance. These primer sets are shown below, along with the sequence of the genes that they are used to analyze. In the gene sequences, the locations of the primers are underlined.

Primers

rpoB (rifampin resistance)

rpoB-F amplification primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3' SEQ. ID NO. 1

rpoB-R amplification primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3' SEQ ID NO. 2

rpoB-5s sequencing primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3' SEQ ID NO. 3

rpoB-3s sequencing primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3' SEQ ID NO. 4

SEQ. ID. NO. 5

```
2161 aaaccgacga catcgaccac ttcggcaacc gccgcctgcg tacggtcggc gagctgatcc
2221 aaaaccagat ccgggtcggc atgtcgcgga tggagcgggt ggtccgggag cggatgacca
2281 cccaggacgt ggaggcgatc acaccgcaga cgttgatcaa catccggccg gtggtcgccg
2341 cgatcaagga gttcttcggc accagccagc tgagccaatt catggaccag aacaacccgc
2401 tgtcggggtt gacccacaag cgccgactgt cggcgctggg gcccggcggt ctgtcacgtg
2461 agcgtgccgg gctggaggtc cgcgacgtgc acccgtcgca ctacggccgg atgtgcccga
2521 tcgaaacccc tgaggggccc aacatcggtc tgatcggctc gctgtcggtg tacgcgcggg
2581 tcaacccgtt cgggttcatc gaaacgccgt accgcaaggt ggtcgacggc gtggttagcg
```

katG (isoniazid resistance)

katG-F amplification primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3' SEQ ID NO. 6

katG-R amplification primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3' SEQ ID NO. 7

katG-5s sequencing primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3' SEQ ID NO. 8

katG-3s sequencing primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3' SEQ ID NO. 9

SEQ ID NO. 10

```
661  gctcggcgat gagcgttaca gcggtaagcg ggatctggag aacccgctgg ccgcggtgca
721  gatggggctg atctacgtga acccggaggg gccgaacggc aacccggacc ccatggccgc
781  ggcggtcgac attcgcgaga cgtttcggcg catggccatg aacgacgtcg aaacagcggc
841  gctgatcgtc ggcggtcaca ctttcggtaa gacccatggc gccggcccgg ccgatctggt
901  cggccccgaa cccgaggctg ctccgctgga gcagatgggc ttgggctgga agagctcgta
961  tggcaccgga accggtaagg acgcgatcac cagcggcatc gaggtcgtat ggacgaacac
1021 cccgacgaaa tgggacaaca gtttcctcga gatcctgtac ggctacgagt gggagctgac
```

1081 gaagagccct gctggcgctt ggcaatacac cgccaaggac ggcgccggtg ccggcaccat
1141 cccggacccg ttcggcgggc cagggcgctc cccgacgatg ctggccactg acctctcgct
1201 gcgggtggat ccgatctatg agcggatcac gcgtcgctgg ctggaacacc ccgaggaatt
1261 ggccgacgag ttcgccaagg cctggtacaa gctgatccac cgagacatgg gtcccgttgc

oxyR-aphC intergenic region (PR)

PR-F amplification primer, 20-mer, bp451-470
5' ACC ACT GCT TTG CCG CCA CC 3' SEQ ID NO. 11
PR-R amplification primer, 20-mer, bp687-668
5' CCG ATG AGA GCG GTG AGC TG 3' SEQ ID NO. 12
PR-5s sequencing primer, 20-mer, bp451-470
5' ACC ACT GCT TTG CCG CCA CC 3' SEQ ID NO. 13
PR-3s sequencing primer, 20-mer, bp687-668
5' CCG ATG AGA GCG GTG AGC TG 3' SEQ ID NO. 14

SEQ ID NO. 15

361 atgccctggg ggtgcaccga gaccggcttc cgaccaccgc tcgccgcaac gtcgactggc
421 tcatatcgag aatgcttgcg gcactgctga accactgctt tgccgccacc gcggcgaacg
481 cgcgaagccc ggccacggcc ggctagcacc tcttggcggc gatgccgata aatatggtgt
541 gatatatcac ctttgcctga cagcgacttc acggcacgat ggaatgtcgc aaccaaatgc
601 attgtccgct ttgatgatga ggagagtcat gccactgcta accattggcg atcaattccc
661 cgcctaccag ctcaccgctc tcatcggcgg tgacctgtcc aaggtcgacg ccaagcagcc
721 cggcgactac ttcaccacta tcaccagtga cgaacaccca ggcaagtggc gggtggtgtt

mabA (isoniazid resistance)

mabA-F amplification primer, 20-mer, bp56-75
5' CCT CGC TGC CCA GAA AGG GA 3' SEQ ID NO. 16
mabA-R amplification primer, 20-mer, bp303-284
5' ATC CCC CGG TTT CCT CCG GT 3' SEQ ID NO. 17
mabA-5s sequencing primer, 20-mer, bp56-75
5' CCT CGC TGC CCA GAA AGG GA 3' SEQ ID NO. 18

mabA-3s sequencing primer, 20-mer, bp303-284

5' ATC CCC CGG TTT CCT CCG GT 3' SEQ ID NO. 19

SEQ ID NO. 20

```
1   agcgcgacat acctgctgcg caattcgtag ggcgtcaata cacccgcagc cagggcctcg
61  ctgcccagaa agggatccgt catggtcgaa gtgtgctgag tcacaccgac aaacgtcacg
121 agcgtaaccc cagtgcgaaa gttcccgccg gaaatcgcag ccacgttacg ctcgtggaca
181 taccgatttc ggcccggccg cggcgagacg ataggttgtc ggggtgactg ccacagccac
241 tgaaggggcc aaacccccat tcgtatcccg ttcagtcctg gttaccggag gaaaccgggg
301 gatcgggctg gcgatcgcac agcggctggc tgccgacggc cacaaggtgg ccgtcaccca
```

rpsL/s12 (streptomycin resistance)

s12-F amplification primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3' SEQ ID NO. 21

s12-R amplification primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3' SEQ ID NO 22

s12-5s sequencing primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3' SEQ ID NO. 23

s12-3s sequencing primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3' SEQ ID NO. 24

SEQ ID NO. 25

```
1   cggtagatgc caaccatcca gcagctggtc cgcaagggtc gtcgggacaa gatcagtaag
61  gtcaagaccg cggctctgaa gggcagcccg cagcgtcgtg gtgtatgcac ccgcgtgtac
121 accaccactc cgaagaagcc gaactcggcg cttcggaagg ttgcccgcgt gaagttgacg
181 agtcaggtcg aggtcacggc gtacattccc ggcgagggcc acaacctgca ggagcactcg
241 atggtgctgg tgcgcggcgg ccgggtgaag gacctgcctg gtgtgcgcta caagatcatc
301 cgcggttcgc tggatacgca gggtgtcaag aaccgcaaac aggcacgcag ccgttacggc
361 gctaagaagg agaagggctg atgccacgca aggggcccgc gcccaagcgt ccgttggtca
```

16S/rrs (streptomycin resistance)

16S-F amplification primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3' SEQ ID NO. 26

16S-R amplification primer, 21-mer, bp147-127

5' CGT CAC CCC ACC AAC AAG CTG 3' SEQ ID NO. 27

16S-5s sequencing primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3' SEQ ID NO. 28

16S-3s sequencing primer, 21-mer, bp147-127

5' CGT CAC CCC ACC AAC AAG CTG 3' SEQ ID NO. 29

SEQ ID NO. 30

```
1   cgtgggtgat ctgccctgca cttcgggata agcctgggaa actgggtcta ataccggata
61  ggaccacggg atgcatgtct tgtggtggaa agcgctttag cggtgtggga tgagcccgcg
121 gcctatcagc ttgttggtgg ggtgacg
```

embB (ethambutol resistance)

embB-F amplification primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3' SEQ ID NO. 31

embB-R amplification primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3 SEQ ID NO. 32

embB-5s sequencing primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3' SEQ ID NO. 33

embB-3s sequencing primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3 SEQ ID NO. 34

SEQ ID NO. 35

```
7741 cggcatgcgc cggctgattc cggcaagctg gcgcaccttc accctgaccg acgccgtggt
7801 gatattcggc ttcctgctct ggcatgtcat cggcgcgaat tcgtcggacg acggctacat
7861 cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgctggtt
7921 cggcagcccg gaggatccct tcggctggta ttacaacctg ctggcgctga tgacccatgt
7981 cagcgacgcc agtctgtgga tgcgcctgcc agacctggcc gccgggctag tgtgctggct
```

pncA (pyrazinamide resistance)

pncA-F amplification primer, 20-mer, bp1-20

5' ATG CGG GCG TTG ATC ATC GT 3' SEQ ID NO. 36

pncA-F amplification primer, 20-mer, bp561-542

5' TCA GGA GCT GCA AAC CAA CT 3' SEQ ID NO. 37

pncA-5s sequencing primer, 20-mer, bp1-20

5' ATG CGG GCG TTG ATC ATC GT 3' SEQ ID NO. 38

pncA-3s sequencing primer, 20-mer, bp561-542

5' TCA GGA GCT GCA AAC CAA CT 3' SEQ ID NO. 39

SEQ ID. NO. 40

```
1   atgcgggcgt tgatcatcgt cgacgtgcag aacgacttct gcgagggtgg ctcgctggcg
61  gtaaccggtg gcgccgcgct ggcccgcgcc atcagcgact acctggccga agcggcggac
121 taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca cttctccggc
181 acaccggact attcctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgcggac
241 ttccatccca gtctggacac gtcggcaatc gaggcggtgt tctacaaggg tgcctacacc
301 ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgccactgct gaattggctg
361 cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc
421 cagacggccg aggacgcggt acgcaatggc ttggccacca gggtgctggt ggacctgaca
481 gcgggtgtgt cggccgatac caccgtcgcc gcgctggagg agatgcgcac cgccagcgtc
541 gagttggttt gcagctcctg a
```

gyrA (fluoroquinilone/ciprofloxacin resistance)

gyrA-F amplification primer, 20-mer, bp2383-2402

5' CAG CTA CAT CGA CTA TGC GA 3' SEQ ID NO. 41

gyrA-R amplification primer, 20-mer, bp2702-2683

5' GGG CTT CGG TGT ACC TCA TC 3' SEQ ID NO. 42

gyrA-5s sequencing primer, 20-mer, bp2383-2402

5' CAG CTA CAT CGA CTA TGC GA 3' SEQ ID NO. 43

gyrA-3s sequencing primer, 20-mer, bp2702-2683

5' GGG CTT CGG TGT ACC TCA TC 3' SEQ ID NO. 44

SEQ ID NO. 45

```
2341 cgaccggatc gaaccggttg acatcgagca ggagatgcag cgcagctaca tcgactatgc
2401 gatgagcgtg atcgtcggcc gcgcgctgcc ggaggtgcgc gacgggctca agcccgtgca
2461 tcgccgggtg ctctatgcaa tgttcgattc cggcttccgc ccggaccgca gccacgccaa
```

2521 gtcggcccgg tcggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat
2581 ctacgacagc ctggtgcgca tggcccagcc ctggtcgctg cgctacccgc tggtggacgg
2641 ccagggcaac ttcggctcgc caggcaatga cccaccggcg gcgatgaggt acaccgaagc
2701 ccggctgacc ccgttggcga tggagatgct gagggaaatc gacgaggaga cagtcgattt

23S (macrolide/azithromycin resistance)

23S-F amplification primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3' SEQ ID NO. 46

23S-R amplification primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3' SEQ ID NO. 47

23S-5s sequencing primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3' SEQ ID NO. 48

23S-3s sequencing primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3' SEQ ID NO. 49

SEQ ID NO. 50

2401 gccccagtaa acggcggtgg taactataac catcctaagg tagcgaaatt ccttgtcggg
2461 taagttccga cctgcacgaa tggcgtaacg acttcccaac tgtctcaacc atagactcgg
2521 cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccggga
2581 ccttcactac aacttggtat tggtgttcgg tacggtttgt gtaggatagg tgggagactt
2641 tgaagcacag acgccagttt gtgtggagtc gttgttgaaa taccactctg atcgtattgg

To facilitate detection of the sequencing products using real-time fluorescence-based electrophoresis apparatus (for example, a Visible Genetics OPENGENE™ sequencer), at least one of the sequencing primers is preferably labeled with a flourescent label. The label is selected for compatibility with the sequencing apparatus employed, and may be, for example, fluorescein or a cyanine dye such as CY5.0 OR CY5.5.

The primers of the invention are suitably packaged in a kit. This kit will contain individually packaged amplification and sequencing primers sets for each resistance gene to be evaluated by the kit. Thus, the kit of the invention includes at least 4 primers (two amplification and two sequencing primers); and preferably includes the primer sets for a plurality of resistance genes, most preferably the primer sets for all ten resistance genes.

The suitable protocol for the utilization of these primer sets in the evaluation of *M. tuberculosis* in clinical samples utilizes PCR amplification, followed by cycle sequencing. DNA for use in the test is obtained from a sample of sputum (100ul-10ml). The sputum sample is processed according to Standard Infectious Disease/Public Health Laboratory practices (Mycobacteriology Bench Manual, Laboratory Services Branch, December 1997, Ontario Ministry of Health). The sputum sample is homogenized, decontaminated and concentrated. Mycobacterial DNA is prepared directly from a portion of the concentrated cell sediment (100-200ul) using standard DNA extraction methods or commercially available kits.

Amplification of the DNA is performed using the amplification primer sets described above. PCR reagents can be prepared for individual reactions, or may be prepared as a master mix which can be used for multiple tests e.g., 10 PCR reactions. Exemplary combinations of reagents are summarized in the following table.

PCR mix		**1 PCR**	**10 PCRs**	**final conc. / PCR**
genomic DNA (~0.5fM)	(20ng/ul)	1.0ul		20ng
10X PCR buffer I		2.5ul	25.0ul	1X
2.5mM dNTP mix	(1:1:1:1)	2.5ul	25.0ul	250uM
DMSO		1.3ul	13.0ul	5%
Taq DNA polymerase	(1U)	0.2ul	2.0ul	1 unit
molecular grade water		16.5ul		165.0ul
MTB gene primers	(10uM)	1.0ul	10.0ul	10pmol per primer
total volume per PCR		25.0ul		

If the master mix as shown in the column labeled 10 PCRs is utilized, the mastermix contains all the necessary PCR reagents other than the genomic DNA. In this example, 24.0ul of the mastermix is added to a PCR tube, that already contains 1.0ul of genomic DNA, prior to the addition of the mineral oil overlay and placement in the thermocycler.

The genomic DNA preparation utilized must be of sufficient quality and integrity for robust and reproducible PCR. Suitable DNA preparation can be obtained using

the Gentra Puregene™ DNA isolation kit. The kit components are appropriate for the isolation of genomic DNA from blood, fresh or frozen tissue, archival material and paraffin-embedded tissue.

Each primer pair is used to amplify a single gene region under the following conditions:

1.	Denaturation	94°C	5 minutes	1 cycle
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35 cycles
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1 cycle
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

After amplification, 2.0ul from the 25.0ul PCR is analysed for purity on a 0.8% agarose gel. Samples displaying single PCR product bands can be used directly for sequence analysis. The yield and purity of the PCR product determines the amount to be used in the subsequent cycle sequencing reaction. Comparable verification of sequencing purity is performed on each of the other amplification products.

Sequence analysis is carried out on the amplified product. The basic procedures and conditions are the same for each region. Accordingly, the invention will be exemplified using the rpoB gene.

For initial sequence analysis of rpoB, the rpoB-5s primer should be used. For confirmatory sequence analysis the rpoB-3s primer should be used. For each template to be sequenced, aliquot 3.0ul of each of the nucleotide termination mixes into four separate tubes marked <A>, <C>, <G> and <T> and store on ice until the sequencing mastermix is prepared.

Cycle sequencing mastermix

rpoB template	2.0ul
10X VGI Sequenace ™ buffer	2.5ul

DMSO	3.5ul
2.5uM dye-sequencing primer	2.0ul
PCR grade water	9.0ul
1:10 diluted Thermosequenase	0.5 ul
total volume	22.0ul

Mix the DMSO and other components in the mastermix well by repeated pipetting (5 times) with a micropipette. Store the mastermix on ice until ready to add to the nucleotide termination mixes.

Add 5.0ul of the mastermix to each of the four marked tubes containing the nucleotide termination mixes.

Add 8.0ul lightweight mineral oil to each of the four marked tubes containing the mastermix and nucleotide termination mixes.

Store on ice until ready to load into the thermocycler.

Parameters for cycle sequencing

1.	Denaturation	94°C	5 minutes	1X
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35X
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1X
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

At the end of the cycle sequencing reaction add 6.0ul of the Stop Loading Dye directly to each of the four tubes to stop the sequencing reaction. The sequencing samples are heated at 95°C for 2 minutes and then placed on ice before loading 2.0ul (from a total volume of 14ul) on the CLIPPER™ sequencer. The remainder of the sequencing reaction can be stored at -20°C for subsequent use.

The CLIPPER™ sequencer is set-up as described in the ***OPENGENE Automated DNA Sequencing System User Manual.*** Run parameters for the CLIPPER™ sequencer are 54°C/ 1300volts/ 0.5sec sampling/35min run/50% laser power. The samples loaded included 2 ul each of the forward and reverse sequencing reaction products for the target gene, differentially labeled, for example with CY5.0 and CY5.5 cyanine dye labels. Once the run is completed, the base-called data is analysed by comparison of the test sequence to the rpoB sequence database in GENELIBRARIAN™. This sequence alignment compares the test sequence to the standard control sequence and allows sequence ambiguities to be assessed. Once edited the test sequence can be screened for antibiotic resistance-associated mutations using GENELIBRARIAN™.

Testing for multiple types of antibiotic-resistance mutations can be carried out using a hierarchical assay, as summarized in Fig. 2. At present molecular biological methods for the detection of *M. tuberculosis* are only performed on AFB smear-positive sputum samples. These methods serve as confirmatory tests for the presence of *M. tuberculosis*. In addition to these molecular biological methods, the culture-based procedures for *M. tuberculosis* detection (BacTec liquid culture, agar plate and slant cultures) are performed in parallel. AFB smear-negative sputum samples are processed with only the culture-based detection procedures (Figure 1).

In the present invention both AFB smear-positive and smear-negative sputum samples can be processed using both culture-based and molecular biological methods. A limitation of the AFB stain methodology is it's limit of detection. If a sputum sample has a mycobacterial concentration of less than 5000 bacteria/ul the AFB stain will be negative. In addition to this is the observation that the decontamination procedure used to prepare the sputum sample usually kills 10-20% of the mycobacteria present. This would suggest that two-thirds of the AFB smear-negative samples potentially contain mycobacteria. In practice 10-20% of the AFB smear-negative samples are culture-positive for *M. tuberculosis* (Ontario Public Health Laboratory). This level of mycobacteria is easily detected by molecular biological methods and is therefore incorporated in the present invention.

The hierarchy proposed incorporates tests that specifically detect M. tuberculosis (rpoB), detect mutations in genes associated with resistance to the "first-line" antibiotics used to treat M. tuberculosis infections (rpoB, katG, rpsL/s12, PR, embB, pncA)

and detect other species of mycobacteria (23S) in the absence of *M. tuberculosis* (Figure 2). Group I analyses are performed before both Group II and Group III. Group I analysis will provide information on the antibiotic resistance status to rifampin (rpoB), isoniazid (katG), streptomycin (rpsL/s12) and azithromycin (23S). In addition the rpoB amplification indicates the presence of *M. tuberculosis* and in the absence of rpoB amplification the 23S sequence allows identification of most of the clinically relevant mycobacterial species. Group II analysis provides information on antibiotic resistance mutations in the "second-line" antibiotics used to treat *M. tuberculosis* infections namely, isoniazid (PR), ethambutol (embB), pyrazinamide (pncA) and ciprofloxacin (gyrA). Group III contains gene targets in which mutations associated with antibiotic resistance are infrequently found. This protocol permits specific gene targets to be examined according to the local treatment procedures since the both antibiotics used to treat *M. tuberculosis* infections, and thus the associated antibiotic resistance mutation patterns, vary geographically. As shown in Figure 2 the culture-based methods are performed in parallel. The molecular biological methods would permit the identification of *M. tuberculosis* from both AFB smear-positive and smear-negative sputum samples and further provide information on the antibiotic resistance profile of these samples well in advance of current culture-based methods. This information would be crucial to the initiation of appropriate and effective antibiotic treatment regimens for *M. tuberculosis* infections.

Examples

A pool of DNA samples from antibiotic-sensitive *M. tuberculosis* isolates was obtained from the LCDC, Health and Welfare Canada. Ottawa, Ontario. Wild-type sequence traces, for all gene targets known to harbor mutations in antibiotic-resistant *M. tuberculosis*, were generated.

A panel of DNA samples from five phenotypic streptomycin-resistant *M. tuberculosis* isolates was obtained from the Public Health Laboratory, Ontario Ministry of Health, Toronto, Ontario. These DNA samples were examined for antibiotic resistance-associated mutations in all 10 antibiotic gene targets listed above. Streptomycin resistance-associated mutations were detected in the rpsL/s12 gene in four isolates. Parallel antibiotic resistance-associated mutations in the rpoB (rifampin), katG (isoniazid), PR (isoniazid),

embB (ethambutol), pncA (pyrazinamide) and gyrA (ciprofloxacin) genes were also identified which underscores the importance of examining all the gene targets for first-line antibiotics used in the treatment of *M. tuberculosis*. A summary of the results is shown in Table 1.

The following references are cited herein and are incorporated herein by reference for all states which allow such incorporation.

[a] **DL Williams et al. (1994). Characterisation of rifampin resistance in pathogenic mycobacteria. Antimicrob Agents Chemother 38: 2380-2386.**

[b] **WH Haas et al. (1997). Molecular analysis of katG gene mutations in strains of Mycobacterium tuberculosis complex from Africa. Antimicrob Agents Chemother 41: 1601-1603.**

[c] **S Sreevatsan et al. (1997). Analysis of the oxyR-ahpC region in isoniazid-resistant and –susceptible Mycobacterium tuberculosis complex organisms recovered from diseased humans and animals in diverse localities. Antimicrob Agents Chemother 41: 600-606.**

[d] **A Telenti et al. (1994). Genotypic assessment of isoniazid and rifampin resistance in Mycobacterium tuberculosis: a blind study at the reference laboratory level. Antimicrob Agents Chemother 35: 719-723.**

[e] **C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of Mycobacterium tuberculosis in Japan. J Appl Microbiol 83: 634-640.**

[f] **C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of Mycobacterium tuberculosis in Japan. J Appl Microbiol 83: 634-640.**

[g] **MA Lety et al. (1997). A single point mutation in the embB gene is responsible for resistance to ethambutol in Mycobacterium smegmatis. Antimicrob Agents Chemother 41: 2629-2633.**

[h] **A Scorpio et al. (1997). Characaterisation of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis. Antimicrob Agents Chemother 41: 540-543.**

C Xu et al. (1996). Fluoroquinilone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant Mycobacterium tuberculosis. J Infect Disease 174: 1127-1130.

KA Nash et al. (1995). Genetic basis of macrolide resistance in Mycobacterium avium isolated from patients with disseminated disease. Antimicrob Agents Chemother 39: 2625-2630.

Table 1

gene (antiblotic)	OPH#1 bp/codon/aa	OPH#2 bp/codon/aa	OPH#3 bp/codon/aa	OPH#4 bp/codon/aa	OPH#11 bp/codon/aa
rpoB (rifampin)	cac526tac, His526Tyr	tcg553ttg, Ser553Leu	cac526gac, His526Asp	tcg553ttg, Ser553Leu	wt
katG.1 (isoniazid)	agc513acc, Ser513Thr	agc513acc, Ser513Thr	agc513acc, Ser513Thr	wt	wt
oxyR-ahpC PR (isoniazid)	g541a	wt	wt	wt	g541a
fabG (isoniazid)	wt	wt	wt	wt	wt
rpsL/s12 (streptomycin)	wt	aag43agg, Lys43Arg	aag43agg, Lys43Arg	aag88agg, Lys88Arg	aag43agg, Lys43Arg
16s/rrs (streptomycin)	wt	wt	wt	wt	wt
embB (ethambutol)	wt	gtc292ttc, val292phe	wt	wt	wt
pncA (pyrazinamide)	tcc65tct, Ser65Ser	wt	att133aat, Ile133Asn	wt	tcc65tct, Ser65Ser
gyrA (ciprofloxacin)	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr
23s (azithromycin)	wt	wt	wt	wt	wt

CLAIMS

1. A method for detection and characterization of *Mycobacterium tuberculosis* present in a sample, comprising the steps of:

(a) obtaining a sputum sample suspected of containing *M. tuberculosis*,

(b) performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the rpoB, katG, rpsL/s12 and 23S genes for the presence of antibiotic-resistance inducing mutations; and

(c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic-resistance inducing mutations.

2. The method of claim 1, wherein the second sequencing procedure evaluates PR, embB pncA and gyrA genes for the presence of antibiotic-resistance mutations.

3. The method of claim 3, further comprising the step of performing a third sequencing procedure when *M. tuberculosis* was detected in step (b) to evaluate 16S/rrs and mabA genes for the presence of antibiotic-resistance mutations.

4. The method of any of claims 1 to 3, wherein the first sequencing procedure for rpoB is performed using amplification primers as set forth in Seq. ID Nos. 1 and 2 and sequencing primers as set forth in Seq. ID. Nos. 3 and 4.

5. The method of any of claims 1 to 4, wherein the first sequencing procedure for katG is performed using amplification primers as set forth in Seq. ID Nos. 6 and 7 and sequencing primers as set forth in Seq. ID. Nos. 8 and 9.

6. The method of any of claims 1 to 5, wherein the first sequencing procedure for rpsL/s12 is performed using amplification primers as set forth in Seq. ID Nos. 21 and 22 and sequencing primers as set forth in Seq. ID. Nos. 23 and 24.

7. The method of any of claims 1 to 6, wherein the second sequencing procedure for 23S is performed using amplification primers as set forth in Seq. ID Nos. 46 and 47 and sequencing primers as set forth in Seq. ID. Nos. 48 and 49.

8. The method of any of claims 1 to 7, wherein the second sequencing procedure for PR is performed using amplification primers as set forth in Seq. ID Nos. 11 and 12 and sequencing primers as set forth in Seq. ID. Nos. 13 and 14.

9. The method of any of claims 1 to 8, wherein the second sequencing procedure for pncA is performed using amplification primers as set forth in Seq. ID Nos. 36 and 37 and sequencing primers as set forth in Seq. ID. Nos. 38 and 39.

10. The method of any of claims 1 to 9, wherein the second sequencing procedure for embB is performed using amplification primers as set forth in Seq. ID Nos. 31 and 32 and sequencing primers as set forth in Seq. ID. Nos. 33 and 34.

11. The method of any of claims 1 to 10, wherein the second sequencing procedure for gyrA is performed using amplification primers as set forth in Seq. ID Nos. 41 and 42 and sequencing primers as set forth in Seq. ID. Nos. 43 and 44.

12. The method of any of claims 2 to 11, wherein the third sequencing procedure for 16S/rrs is performed using amplification primers as set forth in Seq. ID Nos. 26 and 27 and sequencing primers as set forth in Seq. ID. Nos. 28 and 29.

13. The method of any of claims 2 to 12, wherein the third sequencing procedure for mabA is performed using amplification primers as set forth in Seq. ID Nos. 16 and 17 and sequencing primers as set forth in Seq. ID. Nos. 18 and 19.

14. A kit for evaluation of antibiotic-resistance mutations in a sample of *Mycobacterium tuberculosis*, comprising one or more pairs of amplification primers and one or more matched pairs of sequencing primers for amplification and sequencing regions within

the genome of *M. tuberculosis*, characterized in that the amplification and sequencing primer pairs are selected from among:

(a) amplification primers of Seq. ID Nos. 1 and 2 in combination and sequencing primers of Seq. ID Nos. 3 and 4;

(b) amplification primers of Seq. ID Nos. 6 and 7 in combination and sequencing primers of Seq. ID Nos. 8 and 9;

(c) amplification primers of Seq. ID Nos. 11 and 12 in combination and sequencing primers of Seq. ID Nos. 13 and 14;

(d) amplification primers of Seq. ID Nos. 16 and 17 in combination and sequencing primers of Seq. ID Nos. 18 and 19;

(e) amplification primers of Seq. ID Nos. 21 and 22 in combination and sequencing primers of Seq. ID Nos. 23 and 24;

(f) amplification primers of Seq. ID Nos. 26 and 27 in combination and sequencing primers of Seq. ID Nos. 28 and 29;

(g) amplification primers of Seq. ID Nos. 31 and 32 in combination and sequencing primers of Seq. ID Nos. 33 and 34;

(h) amplification primers of Seq. ID Nos. 36 and 37 in combination and sequencing primers of Seq. ID Nos. 38 and 39;

(i) amplification primers of Seq. ID Nos. 41 and 42 in combination and sequencing primers of Seq. ID Nos. 43 and 44; and

(j) amplification primers of Seq. ID Nos. 46 and 47 in combination and sequencing primers of Seq. ID Nos. 48 and 49.

Figure 1

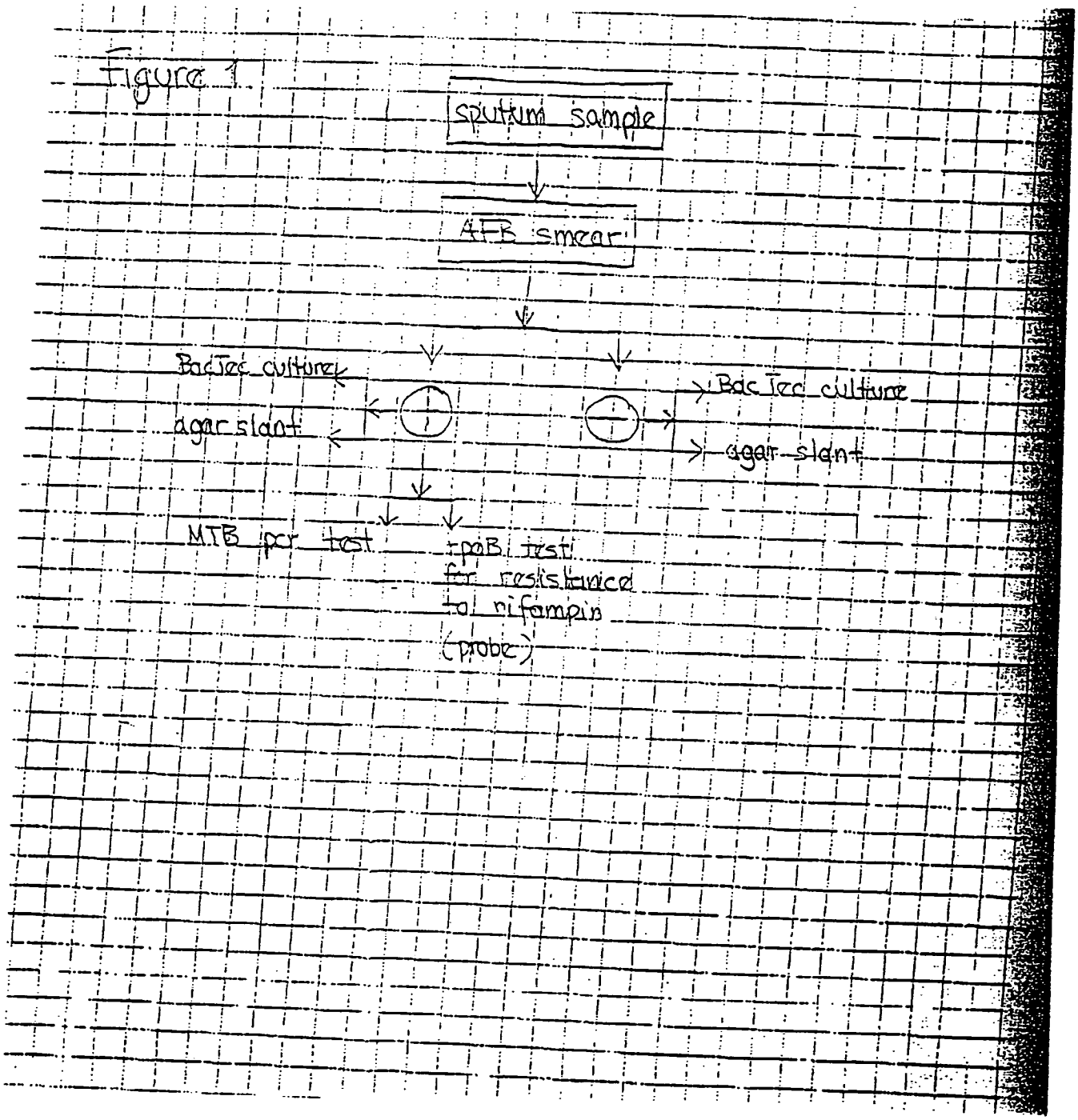
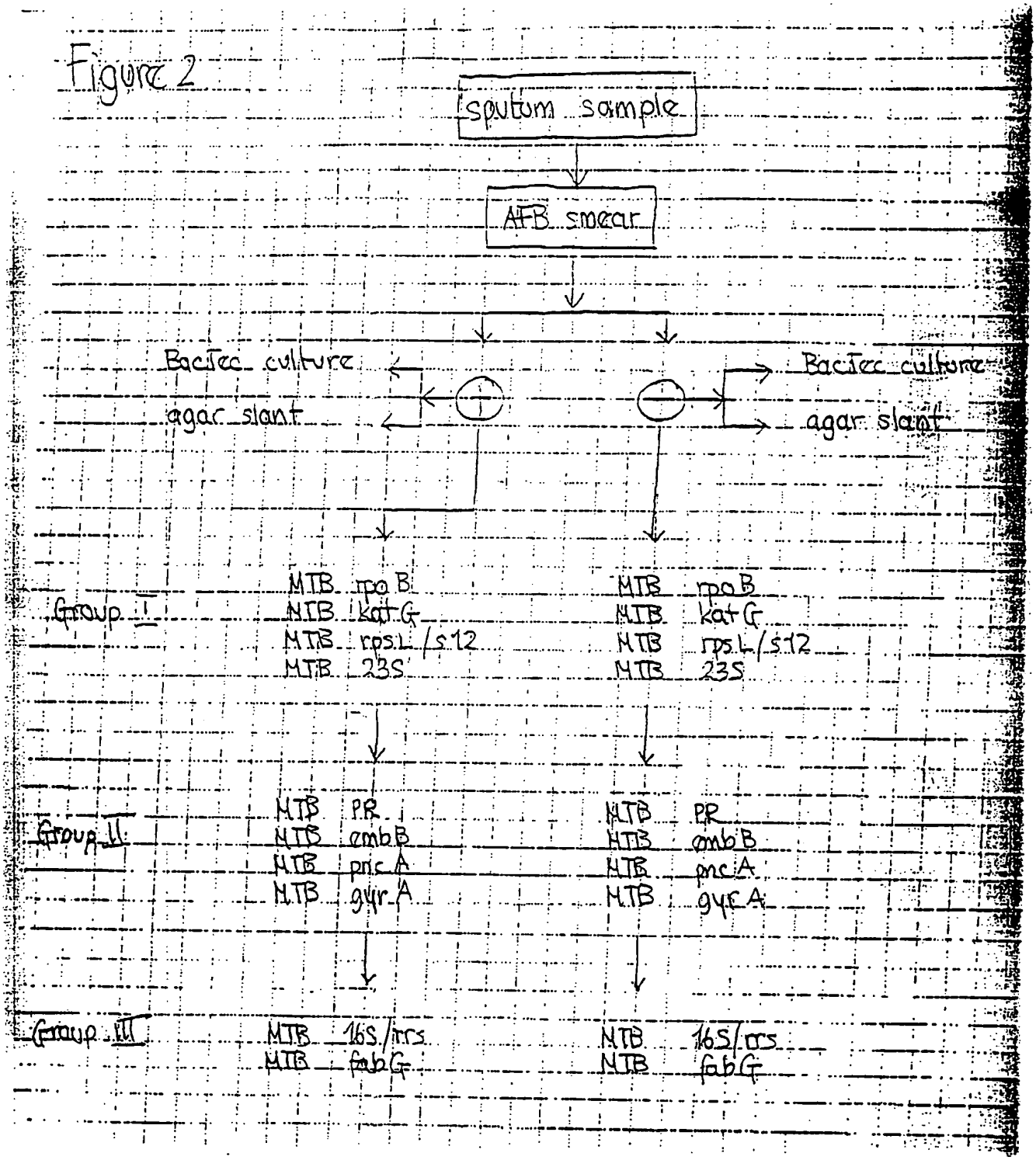


Figure 2



SEQUENCE LISTING

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Shipman, Robert

<120> Method and Kit for the Characterization of Antibiotic-Resistance Mutations in Mycobacterium tuberculosis

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<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 16S-3s sequencing primer

<400> 29
cgtcacccca ccaacaagct g 21

<210> 30
<211> 147
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 16S/rrs (streptomycin resistance)

<400> 30
cgtgggtgat ctgccctgca cttcgggata agcctgggaa actgggtcta ataccggata 60
ggaccacggg atgcatgtct tgtggtggaa agcgctttag cggtgtggga tgagcccgcg 120
gcctatcagc ttgttggtgg ggtgacg 147

<210> 31
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB-F amplification primer

<400> 31
cggcaagctg gcgcaccttc a 21

<210> 32
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB-R amplification primer

<400> 32
agccagcaca ctagcccggc g 21

<210> 33
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB-5s seuqencing primer

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<210> 34
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB-3s sequencing primer

<400> 34
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<210> 35
<211> 300
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB (ethambutol resistance)

<400> 35
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cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgctggtt 180
cggcagcccg gaggatccct tcggctggta ttacaacctg ctggcgctga tgacccatgt 240
cagcgacgcc agtctgtgga tgcgcctgcc agacctggcc gccgggctag tgtgctggct 300

<210> 36
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<212> DNA
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<220>
<223> pncA-F amplification primer

<400> 36
atgcgggcgt tgatcatcgt 20

<210> 37
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-F amplification primer

<400> 37
tcaggagctg caaaccaact 20

<210> 38
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-5s sequencing primer

<400> 38
atgcgggcgt tgatcatcgt 20

<210> 39
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-3s sequencing primer

<400> 39
tcaggagctg caaaccaact 20

<210> 40
<211> 561
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA (pyrazinamide resistance)

<400> 40
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gtaaccggtg gcgccgcgct ggcccgcgcc atcagcgact acctggccga agcggcggac 120
taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca cttctccggc 180
acaccggact attcctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgcggac 240
ttccatccca gtctggacac gtcggcaatc gaggcggtgt tctacaaggg tgcctacacc 300

ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgccactgct gaattggctg 360
cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc 420
cagacggccg aggacgcggt acgcaatggc ttggccacca gggtgctggt ggacctgaca 480
gcgggtgtgt cggccgatac caccgtcgcc gcgctggagg agatgcgcac cgccagcgtc 540
gagttggttt gcagctcctg a 561

<210> 41
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA-F amplification primer

<400> 41
cagctacatc gactatgcga 20

<210> 42
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA-R amplification primer

<400> 42
gggcttcggt gtacctcatc 20

<210> 43
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA-5s sequencing primer

<400> 43
cagctacatc gactatgcga 20

<210> 44
<211> 20

<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA-3s sequencing primer

<400> 44
gggcttcggt gtacctcatc 20

<210> 45
<211> 420
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA (fluoroquinilone/ciprofloxacin resistance)

<400> 45
cgaccggatc gaaccggttg acatcgagca ggagatgcag cgcagctaca tcgactatgc 60
gatgagcgtg atcgtcggcc gcgcgctgcc ggaggtgcgc gacgggctca agcccgtgca 120
tcgccgggtg ctctatgcaa tgttcgattc cggcttccgc ccggaccgca gccacgccaa 180
gtcggcccgg tcggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat 240
ctacgacagc ctggtgcgca tggcccagcc ctggtcgctg cgctacccgc tggtggacgg 300
ccagggcaac ttcggctcgc caggcaatga cccaccggcg gcgatgaggt acaccgaagc 360
ccggctgacc ccgttggcga tggagatgct gagggaaatc gacgaggaga cagtcgattt 420

<210> 46
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S-F amplification primer

<400> 46
cgaaattcct tgtcgggtaa 20

<210> 47
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S-R amplification primer

<400> 47
gtatttcaac aacgactcca 20

<210> 48
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S-5s sequencing primer

<400> 48
cgaaattcct tgtcgggtaa 20

<210> 49
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S-3s sequencing primer

<400> 49
gtatttcaac aacgactcca 20

<210> 50
<211> 300
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S (macrolide/azithromycin resistance)

<400> 50
gccccagtaa acggcggtgg taactataac catcctaagg tagcgaaatt ccttgtcggg 60
taagttccga cctgcacgaa tggcgtaacg acttcccaac tgtctcaacc atagactcgg 120
cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccggga 180
ccttcactac aacttggtat tggtgttcgg tacggtttgt gtaggatagg tgggagactt 240
tgaagcacag acgccagttt gtgtggagtc gttgttgaaa taccactctg atcgtattgg 300

INTERNATIONAL SEARCH REPORT

Int .tional Application No
PCT/CA 99/01177

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAPUR V ET AL: "Application of automated DNA sequence analysis for mycobacterium species identification and detection of mutations associated with antibiotic resistance in Mycobacterium tuberculosis" ABSTRACTS OF THE INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 34, 1994, page 163 XP000901974 see abstract D71 ---	1-3
X	SUZUKI Y ET AL: "Detection of kanamycin-resistant Mycobacterium tuberculosis by identifying mutations in the 16SrRNA gene" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 36, no. 5, May 1998 (1998-05), pages 1220-5, XP000901934 the whole document --- -/--	1-3

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 April 2000	28/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

Int. ational Application No
PCT/CA 99/01177

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HONORE N ET AL: "Streptomycin resistance in mycobacteria" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 38, no. 2, February 1994 (1994-02), pages 238-42, XP000901931 page 239, paragraph 2 ---	1-3
Y	SCORPIO A ET AL: "Characterization of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 41, no. 3, March 1997 (1997-03), pages 540-543, XP000901990 page 540 -page 542, paragraph 4 ---	1-3
Y	ALANGADEN GJ ET AL: "Mechanism of resistance to amikacin and kanamycin in Mycobacterium tuberculosis" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 42, no. 5, May 1998 (1998-05), pages 1295-97, XP000901991 the whole document ---	1-3
Y	HEYM B ET AL: "IMPLICATIONS OF MULTIDRUG RESISTANCE FOR THE FUTURE OF SHORT-COURSE CHEMOTHERAPY OF TUBERCULOSIS:A MOLUCULAR STUDY" LANCET THE,GB,LANCET LIMITED. LONDON, vol. 344, no. 8918, 30 July 1994 (1994-07-30), pages 293-298, XP002039609 ISSN: 0140-6736 the whole document ---	1-3
Y	WO 97 23650 A (DUNN JAMES M ;LEUSHNER JAMES (CA); STEVENS JOHN K (CA); VISIBLE GE) 3 July 1997 (1997-07-03) page 10, paragraph 1 -page 11, paragraph 4; example 7 ---	1-3
A	WO 95 33074 A (MAYO FOUNDATION ;HOFFMANN LA ROCHE (US)) 7 December 1995 (1995-12-07) page 3, paragraph 3 -page 6, paragraph 3 ---	1-3
A	WO 95 33851 A (INNOGENETICS NV ;BEENHOUWER HANS DE (BE); PORTAELS FRANCOISE (BE);) 14 December 1995 (1995-12-14) claims 2,22 ---	1-3

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INTERNATIONAL SEARCH REPORT

Int. tional Application No
PCT/CA 99/01177

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	NUESCA D ET AL: "RAPID DETECTION OF ANTIBIOTIC RESISTANCE-ASSOCIATED MUTATIONS IN 10 GENE TARGETS IN MYCOBACTERIUM TUBERCULOSIS USING THE OPENGENE(R) SYSTEM " ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 99, 30 May 1999 (1999-05-30) - 3 June 1999 (1999-06-03), page 636 XP000891874 see abstract U-13 -----	1-3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir: tional Application No
PCT/CA 99/01177

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9723650 A	03-07-1997	US	5834189 A	10-11-1998
		AU	1426297 A	17-07-1997
		CA	2239896 A	03-07-1997
		EP	0870059 A	14-10-1998
		WO	9741257 A	06-11-1997
		US	5888736 A	30-03-1999
		US	5981186 A	09-11-1999
		US	6007983 A	28-12-1999
WO 9533074 A	07-12-1995	US	5643723 A	01-07-1997
		AU	2605795 A	21-12-1995
WO 9533851 A	14-12-1995	AU	703947 B	01-04-1999
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		CZ	9603612 A	16-07-1997
		EP	0771360 A	07-05-1997
		JP	10500857 T	27-01-1998

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International Bureau

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(81) Designated States *(national)*: AU, CA, JP, US.

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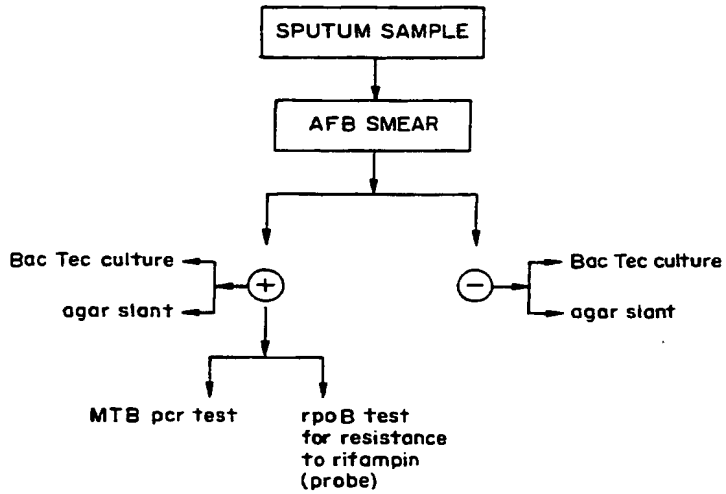
see PCT Gazette No. 28/2001 of 12 July 2001, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD AND KIT FOR THE CHARACTERIZATION OF ANTIBIOTIC-RESISTANCE MUTATIONS IN *MYCOBACTERIUM TUBERCULOSIS*



(57) Abstract: Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the rpoB (rifampin), katG (isoniazid), oxyR-ahpC PR (isoniazid), mabA (isoniazid), rpsL/s12 (streptomycin), 16S/rrs (streptomycin), embB (ethambutol), pncA (pyrazinamide), gyrA (ciprofloxacin) and 23S (azithromycin) genes of *Mycobacterium tuberculosis*. These primers can be used in a method for detection and characterization of *Mycobacterium tuberculosis* present in a sample. The method includes the steps of obtaining a sputum sample suspected of containing M. tuberculosis, performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the rpoB, katG, rpsL/s12 and 23S genes for the presence of antibiotic-resistance inducing mutations; and (c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic-resistance inducing mutations.

WO 00/36142 A1

METHOD AND KIT FOR THE CHARACTERIZATION OF ANTIBIOTIC-RESISTANCE MUTATIONS IN MYCOBACTERIUM TUBERCULOSIS

DESCRIPTION

Field of the Invention

This application relates to a method and kit for the characterization of antibiotic resistance mutations in *Mycobacterium tuberculosis*, and particularly to the evaluation of such mutations in clinical samples.

Background of the Invention

M. tuberculosis can be resistant to all antibiotics that are currently used to treat tuberculosis patients. Antibiotic resistance is due to acquired point mutations in target genes in the genome of *M. tuberculosis*. These point mutations render the organism insensitive to the action of the antibiotic by preventing it's uptake or activation, or by altering the antibiotic target. The observed antibiotic resistance in *M. tuberculosis* is not due to an episome-encoded resistance gene transferred from one strain to another and, like other bacteria, is single-step (one point mutation), high-level resistance.

Rapid and accurate detection of antibiotic resistance in *Mycobacterium tuberculosis* in sputum samples would greatly improve both patient treatment and outcome. Presently, analysis of *M. tuberculosis* is carried out on DNA recovered from sputum samples handled according to Standard Infectious Disease/Public Health Laboratory practices. The sputum sample is decontaminated and a cell sediment isolated. This cell sediment is the sample source for all routine procedures used in the detection and isolation of *M. tuberculosis*. Portions of this sample are used in BacTec cultures for selective growth of *M. tuberculosis*, agar plate/agar slant cultures for M. tuberculosis, acid-fast bacilli (AFB) smears for mycobacteria detection and molecular biological methods for the detection of M. tuberculosis and atypical mycobacteria. (See Fig. 1)

Mycobacterial DNA is prepared directly from the decontaminated sputum cell sediments according to standard procedures and this mycobacterial DNA is used in the various molecular biological detection procedures. The methods presently in use for the detection of *M. tuberculosis* are either PCR-based or probe-based. These tests are used

primarily on AFB smear-positive samples. Since the presence of M. tuberculosis has already been established by the AFB smear, these tests are used primarily in a confirmatory capacity as opposed to a diagnostic capacity. Furthermore, these tests provide no information on the potential antibiotic resistance of these *M. tuberculosis* samples.

Below is a list of antibiotics used to treat *M. tuberculosis* infections. The gene target of the specific antibiotic and regions associated with antibiotic resistance are listed, if known. The references on which the codon assignments are based are listed at the end of the application.

1.	Rifampin	rpoB gene	codon 507-533a
2.	Isoniazid	katG gene	codon 275/315/328b
3.	Isoniazid	mabA gene	unknown c
4.	Isoniazid	oxyR-ahpC intergenic region (PR)	nucleotides -48 to +33
5.	Azithromycin	23S rRNA sequence	nucleotide 2568A e
6.	Pyrazinamide	pncA gene	codon 47/85 f
7.	Ethambutol	embB gene	codon 306 g
8.	Streptomycin	rpsL/s12 gene	codon 43/88 h
9.	Streptomycin	16S/rrs sequence	nucleotides 491, 512, 516, 513, 903, 904
10.	Ciprofloxacin	gyrA gene	codon 88-95 j

Probe-based tests do exist for the determination of rifampin resistance in *M. tuberculosis* (line probe assay-InnoTek), but these probes rely on prior knowledge of antibiotic resistance-associated mutations in the rpoB gene. Mutations outside the region covered by the probe or new mutations not included in the probe cocktail could still confer resistance, but would not be detected using this product in it's present form.

Thus. there remains a need for a method for detecting antibiotic-resistance mutations in clinical M. tuberculosis sputum samples which is capable of detecting mutations in all of the gene targets which confer antibiotic resistance. It is an object of the present invention to provide such a method. It is a further object of this invention to provide amplification and cycle sequencing primer sets, and kits containing such primer sets, for use in the characterization of antibiotic resistance mutations in M. tuberculosis.

Summary of Invention

Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the rpoB (rifampin), katG (isoniazid), oxyR-ahpC PR (isoniazid), mabA (isoniazid), rpsL/s12 (streptomycin), 16S/rrs (streptomycin), embB (ethambutol), pncA (pyrazinamide), gyrA (ciprofloxacin) and 23S (azithromycin) genes. Using these primer sets and the OPENGENE™ automated DNA sequencing system, a protocol has been developed which permits both the rapid detection of *M. tuberculosis* and the identification of antibiotic resistance-associated mutations in a series of gene targets. The present invention uses a series of tests designed to detect antibiotic resistance-associated mutation in all gene targets for all antibiotics presently in use for the treatment of tuberculosis. The tests are employed in a hierarchical manner on both AFB smear-positive or smear-negative samples to determine both the presence and antibiotic-resistance of *M. tuberculosis* in a given sample. This method permits the simultaneous determination of *M. tuberculosis* presence in a sample and the antibiotic-resistance profile to an entire panel of antibiotics. Standard methods require from 2-6 weeks to culture M. tuberculosis and additional time to establish antibiotic resistance. Although DNA sequence-based (genotypic) tests are not intended to replace the traditional culture-based (phenotypic) methods, these tests do represent a rapid, sensitive and accurate protocol which provides clinicians with valuable information regarding antibiotic treatment options within days as opposed to weeks.

Brief Description to the Figures

Fig. 1 shows known testing protocols for M. tuberculosis; and

Fig. 2 shows a hierarchical assay scheme for evaluating M. tuberculosis type in accordance with the invention.

Detailed Description of the Invention

In accordance with the invention, regions of the genome of *M. tuberculosis* associated with antibiotic resistance are amplified and sequenced using specifically designed amplification and sequencing primers. Various techniques for amplification are known, including the basic PCR amplification techniques described in US Patent No. 4,683,202, which is incorporated herein by reference. Similarly, various techniques for sequencing are know, some of which require prior amplification and some of which do not. Included among known sequencing techniques are those disclosed in US Patents Nos. 5,834,189 and 5,789,168, which are incorporated herein by reference. The primers of the invention can be used in any of these sequencing formats, although the invention is exemplified below using separate amplification and cycle-sequencing steps.

In theory, the selection of primers to amplify and sequence a known region of interest should be straightforward. In fact, however, because of the possibility of primer binding to other sites, complications arising from secondary structure, and other factors which are not fully understood, some primers perform better than others for amplification and sequencing of the same region of interest. The present invention provides primers which have been optimized for the amplification and sequencing of regions associated with each of the ten known types of antibiotic resistance. These primer sets are shown below, along with the sequence of the genes that they are used to analyze. In the gene sequences, the locations of the primers are underlined.

Primers

rpoB (rifampin resistance)

rpoB-F amplification primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3' SEQ. ID NO. 1

rpoB-R amplification primer. 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3' SEQ ID NO. 2

rpoB-5s sequencing primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3' SEQ ID NO. 3

rpoB-3s sequencing primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3' SEQ ID NO. 4

SEQ. ID. NO. 5

2161 aaaccgacga catcgaccac ttcggcaacc gccgcctgcg tacggtcggc gagctgatcc
2221 aaaaccagat ccgggtcggc atgtcgcgga tggagcgggt ggtccgggag cggatgacca
2281 cccaggacgt ggaggcgatc acaccgcaga cgttgatcaa catccggccg gtggtcgccg
2341 cgatcaagga gttcttcggc accagccagc tgagccaatt catggaccag aacaacccgc
2401 tgtcggggtt gacccacaag cgccgactgt cggcgctggg gcccggcggt ctgtcacgtg
2461 agcgtgccgg gctggaggtc cgcgacgtgc acccgtcgca ctacggccgg atgtgcccga
2521 tcgaaacccc tgaggggccc aacatcggtc tgatcggctc gctgtcggtg tacgcgcggg
2581 tcaacccgtt cgggttcatc gaaacgccgt accgcaaggt ggtcgacggc gtggttagcg

katG (isoniazid resistance)

katG-F amplification primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3' SEQ ID NO. 6

katG-R amplification primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3' SEQ ID NO. 7

katG-5s sequencing primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3' SEQ ID NO. 8

katG-3s sequencing primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3' SEQ ID NO. 9

SEQ ID NO. 10

661 gctcggcgat gagcgttaca gcggtaagcg ggatctggag aacccgctgg ccgcggtgca
721 gatggggctg atctacgtga acccggaggg gccgaacggc aacccggacc ccatggccgc
781 ggcggtcgac attcgcgaga cgtttcggcg catggccatg aacgacgtcg aaacagcggc
841 gctgatcgtc ggcggtcaca ctttcggtaa **gacccatggc** gccggcccgg ccgatctggt
901 cggccccgaa cccgaggctg ctccgctgga gcagatgggc ttgggctgga agagctcgta
961 tggcaccgga accggtaagg acgcgatcac **cagcggcatc** gaggtcgtat ggacgaacac
1021 cccgacgaaa **tgggacaaca** gtttcctcga gatcctgtac ggctacgagt gggagctgac

1081 gaagagccct gctggcgctt ggcaatacac cgccaaggac ggcgccggtg ccggcaccat

1141 cccggacccg ttcggcgggc cagggcgctc cccgacgatg ctggccactg acctctcgct

1201 gcgggtggat ccgatctatg agcggatcac gcgtcgctgg ctggaacacc ccgaggaatt

1261 ggccgacgag ttcgccaagg cctggtacaa gctgatccac cgagacatgg gtcccgttgc

oxyR-aphC intergenic region (PR)

PR-F amplification primer, 20-mer, bp451-470

5' ACC ACT GCT TTG CCG CCA CC 3' SEQ ID NO. 11

PR-R amplification primer, 20-mer, bp687-668

5' CCG ATG AGA GCG GTG AGC TG 3' SEQ ID NO. 12

PR-5s sequencing primer, 20-mer, bp451-470

5' ACC ACT GCT TTG CCG CCA CC 3' SEQ ID NO. 13

PR-3s sequencing primer, 20-mer, bp687-668

5' CCG ATG AGA GCG GTG AGC TG 3' SEQ ID NO. 14

SEQ ID NO. 15

361 atgccctggg ggtgcaccga gaccggcttc cgaccaccgc tcgccgcaac gtcgactggc

421 tcatatcgag aatgcttgcg gcactgctga accactgctt tgccgccacc gcggcgaacg

481 cgcgaagccc ggccacggcc ggctagcacc tcttggcggc gatgccgata aatatggtgt

541 gatatatcac ctttgcctga cagcgacttc acggcacgat ggaatgtcgc aaccaaatgc

601 attgtccgct ttgatgatga ggagagtcat gccactgcta accattggcg atcaattccc

661 cgcctaccag ctcaccgctc tcatcggcgg tgacctgtcc aaggtcgacg ccaagcagcc

721 cggcgactac ttcaccacta tcaccagtga cgaacaccca ggcaagtggc gggtggtgtt

mabA (isoniazid resistance)

mabA-F amplification primer, 20-mer, bp56-75

5' CCT CGC TGC CCA GAA AGG GA 3' SEQ ID NO. 16

mabA-R amplification primer, 20-mer, bp303-284

5' ATC CCC CGG TTT CCT CCG GT 3' SEQ ID NO. 17

mabA-5s sequencing primer, 20-mer, bp56-75

5' CCT CGC TGC CCA GAA AGG GA 3' SEQ ID NO. 18

mabA-3s sequencing primer, 20-mer, bp303-284

5' ATC CCC CGG TTT CCT CCG GT 3' SEQ ID NO. 19

SEQ ID NO. 20

1 agcgcgacat acctgctgcg caattcgtag ggcgtcaata cacccgcagc cagggcctcg

61 ctgcccagaa agggatccgt catggtcgaa gtgtgctgag tcacaccgac aaacgtcacg

121 agcgtaaccc cagtgcgaaa gttcccgccg gaaatcgcag ccacgttacg ctcgtggaca

181 taccgatttc ggcccggccg cggcgagacg ataggttgtc ggggtgactg ccacagccac

241 tgaaggggcc aaacccccat tcgtatcccg ttcagtcctg gttaccggag gaaaccgggg

301 gatcgggctg gcgatcgcac agcggctggc tgccgacggc cacaaggtgg ccgtcaccca

rpsL/s12 (streptomycin resistance)

s12-F amplification primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3' SEQ ID NO. 21

s12-R amplification primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3' SEQ ID NO 22

s12-5s sequencing primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3' SEQ ID NO. 23

s12-3s sequencing primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3' SEQ ID NO. 24

SEQ ID NO. 25

1 cggtagatgc caaccatcca gcagctggtc cgcaagggtc gtcgggacaa gatcagtaag

61 gtcaagaccg cggctctgaa gggcagcccg cagcgtcgtg gtgtatgcac ccgcgtgtac

121 accaccactc cgaagaagcc gaactcggcg cttcggaagg ttgcccgcgt gaagttgacg

181 agtcaggtcg aggtcacggc gtacattccc ggcgagggcc acaacctgca ggagcactcg

241 atggtgctgg tgcgcggcgg ccgggtgaag gacctgcctg gtgtgcgcta caagatcatc

301 cgcggttcgc tggatacgca gggtgtcaag aaccgcaaac aggcacgcag ccgttacggc

361 gctaagaagg agaagggctg atgccacgca aggggcccgc gcccaagcgt ccgttggtca

16S/rrs (streptomycin resistance)

16S-F amplification primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3' SEQ ID NO. 26

16S-R amplification primer, 21-mer, bp147-127

5' CGT CAC CCC ACC AAC AAG CTG 3' SEQ ID NO. 27

16S-5s sequencing primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3' SEQ ID NO. 28

16S-3s sequencing primer, 21-mer, bp147-127

5' CGT CAC CCC ACC AAC AAG CTG 3' SEQ ID NO. 29

SEQ ID NO. 30

1 cgtgggtgat ctgccctgca cttcgggata agcctgggaa actgggtcta taccggata

61 ggaccacggg atgcatgtct tgtggtggaa agcgctttag cggtgtggga tgagcccgcg

121 gcctatcagc ttgttggtgg ggtgacg

embB (ethambutol resistance)

embB-F amplification primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3' SEQ ID NO. 31

embB-R amplification primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3 SEQ ID NO. 32

embB-5s sequencing primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3' SEQ ID NO. 33

embB-3s sequencing primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3 SEQ ID NO. 34

SEQ ID NO. 35

7741 cggcatgcgc cggctgattc cggcaagctg gcgcaccttc accctgaccg acgccgtggt

7801 gatattcggc ttcctgctct ggcatgtcat cggcgcgaat tcgtcggacg acggctacat

7861 cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgctggtt

7921 cggcagcccg gaggatccct tcggctggta ttacaacctg ctggcgctga tgacccatgt

7981 cagcgacgcc agtctgtgga tgcgcctgcc agacctggcc gccgggctag tgtgctggct

pncA (pyrazinamide resistance)

pncA-F amplification primer, 20-mer, bp1-20

5' ATG CGG GCG TTG ATC ATC GT 3' SEQ ID NO. 36

pncA-F amplification primer, 20-mer, bp561-542
5' TCA GGA GCT GCA AAC CAA CT 3' SEQ ID NO. 37
pncA-5s sequencing primer, 20-mer, bp1-20
5' ATG CGG GCG TTG ATC ATC GT 3' SEQ ID NO. 38
pncA-3s sequencing primer, 20-mer, bp561-542
5' TCA GGA GCT GCA AAC CAA CT 3' SEQ ID NO. 39

SEQ ID. NO. 40

1 atgcgggcgt tgatcatcgt cgacgtgcag aacgacttct gcgagggtgg ctcgctggcg
61 gtaaccgggtg gcgccgcgct ggcccgcgcc atcagcgact acctggccga agcggcggac
121 taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca cttctccggc
181 acaccggact attcctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgcggac
241 ttccatccca gtctggacac gtcggcaatc gaggcggtgt tctacaaggg tgcctacacc
301 ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgccactgct gaattggctg
361 cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc
421 cagacggccg aggacgcggt acgcaatggc ttggccacca gggtgctggt ggacctgaca
481 gcgggtgtgt cggccgatac caccgtcgcc gcgctggagg agatgcgcac cgccagcgtc
541 gagttggttt gcagctcctg a

gyrA (fluoroquinilone/ciprofloxacin resistance)
gyrA-F amplification primer, 20-mer, bp2383-2402
5' CAG CTA CAT CGA CTA TGC GA 3' SEQ ID NO. 41
gyrA-R amplification primer, 20-mer, bp2702-2683
5' GGG CTT CGG TGT ACC TCA TC 3' SEQ ID NO. 42
gyrA-5s sequencing primer, 20-mer, bp2383-2402
5' CAG CTA CAT CGA CTA TGC GA 3' SEQ ID NO. 43
gyrA-3s sequencing primer, 20-mer, bp2702-2683
5' GGG CTT CGG TGT ACC TCA TC 3' SEQ ID NO. 44

SEQ ID NO. 45

2341 cgaccggatc gaaccggttg acatcgagca ggagatgcag cgcagctaca tcgactatgc
2401 gatgagcgtg atcgtcggcc gcgcgctgcc ggaggtgcgc gacgggctca agcccgtgca
2461 tcgccgggtg ctctatgcaa tgttcgattc cggcttccgc ccggaccgca gccacgccaa

2521 gtcggcccgg tcggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat

2581 ctacgacagc ctggtgcgca tggcccagcc ctggtcgctg cgctacccgc tggtggacgg

2641 ccagggcaac ttcggctcgc caggcaatga cccaccggcg gcgatgaggt acaccgaagc

2701 ccggctgacc ccgttggcga tggagatgct gagggaaatc gacgaggaga cagtcgattt

23S (macrolide/azithromycin resistance)

23S-F amplification primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3' SEQ ID NO. 46

23S-R amplification primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3' SEQ ID NO. 47

23S-5s sequencing primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3' SEQ ID NO. 48

23S-3s sequencing primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3' SEQ ID NO. 49

SEQ ID NO. 50

2401 gccccagtaa acggcggtgg taactataac catcctaagg tagcgaaatt ccttgtcggg

2461 taagttccga cctgcacgaa tggcgtaacg acttcccaac tgtctcaacc atagactcgg

2521 cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccggga

2581 ccttcactac aacttggtat tggtgttcgg tacggtttgt gtaggatagg tgggagactt

2641 tgaagcacag acgccagttt gtgtggagtc gttgttgaaa taccactctg atcgtattgg

To facilitate detection of the sequencing products using real-time fluorescence-based electrophoresis apparatus (for example, a Visible Genetics OPENGENE™ sequencer), at least one of the sequencing primers is preferably labeled with a flourescent label. The label is selected for compatibility with the sequencing apparatus employed, and may be, for example, fluorescein or a cyanine dye such as CY5.0 OR CY5.5.

The primers of the invention are suitably packaged in a kit. This kit will contain individually packaged amplification and sequencing primers sets for each resistance gene to be evaluated by the kit. Thus, the kit of the invention includes at least 4 primers (two amplification and two sequencing primers), and preferably includes the primer sets for a plurality of resistance genes, most preferably the primer sets for all ten resistance genes.

The suitable protocol for the utilization of these primer sets in the evaluation of *M. tuberculosis* in clinical samples utilizes PCR amplification, followed by cycle sequencing. DNA for use in the test is obtained from a sample of sputum (100ul-10ml). The sputum sample is processed according to Standard Infectious Disease/Public Health Laboratory practices (Mycobacteriology Bench Manual, Laboratory Services Branch, December 1997, Ontario Ministry of Health). The sputum sample is homogenized, decontaminated and concentrated. Mycobacterial DNA is prepared directly from a portion of the concentrated cell sediment (100-200ul) using standard DNA extraction methods or commercially available kits.

Amplification of the DNA is performed using the amplification primer sets described above. PCR reagents can be prepared for individual reactions, or may be prepared as a master mix which can be used for multiple tests e.g., 10 PCR reactions. Exemplary combinations of reagents are summarized in the following table.

PCR mix	**1 PCR**	**10 PCRs**	**final conc. / PCR**
genomic DNA (20ng/ul)	1.0ul		20ng (~0.5fM)
10X PCR buffer I	2.5ul	25.0ul	1X
2.5mM dNTP mix (1:1:1:1)	2.5ul	25.0ul	250uM
DMSO	1.3ul	13.0ul	5%
Taq DNA polymerase (1U)	0.2ul	2.0ul	1 unit
molecular grade water	16.5ul	165.0ul	
MTB gene primers (10uM)	1.0ul	10.0ul	10pmol per primer
total volume per PCR	25.0ul		

If the master mix as shown in the column labeled 10 PCRs is utilized, the mastermix contains all the necessary PCR reagents other than the genomic DNA. In this example, 24.0ul of the mastermix is added to a PCR tube, that already contains 1.0ul of genomic DNA, prior to the addition of the mineral oil overlay and placement in the thermocycler.

The genomic DNA preparation utilized must be of sufficient quality and integrity for robust and reproducible PCR. Suitable DNA preparation can be obtained using

the Gentra Puregene™ DNA isolation kit. The kit components are appropriate for the isolation of genomic DNA from blood, fresh or frozen tissue, archival material and paraffin-embedded tissue.

Each primer pair is used to amplify a single gene region under the following conditions:

1.	Denaturation	94°C	5 minutes	1 cycle
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35 cycles
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1 cycle
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

After amplification, 2.0ul from the 25.0ul PCR is analysed for purity on a 0.8% agarose gel. Samples displaying single PCR product bands can be used directly for sequence analysis. The yield and purity of the PCR product determines the amount to be used in the subsequent cycle sequencing reaction. Comparable verification of sequencing purity is performed on each of the other amplification products.

Sequence analysis is carried out on the amplified product. The basic procedures and conditions are the same for each region. Accordingly, the invention will be exemplified using the rpoB gene.

For initial sequence analysis of rpoB, the rpoB-5s primer should be used. For confirmatory sequence analysis the rpoB-3s primer should be used. For each template to be sequenced, aliquot 3.0ul of each of the nucleotide termination mixes into four separate tubes marked <A>, <C>, <G> and <T> and store on ice until the sequencing mastermix is prepared.

Cycle sequencing mastermix

rpoB template	2.0ul
10X VGI Sequenace (tm) buffer	2.5ul

DMSO		3.5ul
2.5uM dye-sequencing primer	2.0ul	
PCR grade water		9.0ul
1:10 diluted Thermosequenase	0.5 ul	
total volume		22.0ul

Mix the DMSO and other components in the mastermix well by repeated pipetting (5 times) with a micropipette. Store the mastermix on ice until ready to add to the nucleotide termination mixes.

Add 5.0ul of the mastermix to each of the four marked tubes containing the nucleotide termination mixes.

Add 8.0ul lightweight mineral oil to each of the four marked tubes containing the mastermix and nucleotide termination mixes.

Store on ice until ready to load into the thermocycler.

Parameters for cycle sequencing

1.	Denaturation	94°C	5 minutes	1X
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35X
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1X
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

At the end of the cycle sequencing reaction add 6.0ul of the Stop Loading Dye directly to each of the four tubes to stop the sequencing reaction. The sequencing samples are heated at 95°C for 2 minutes and then placed on ice before loading 2.0ul (from a total volume of 14ul) on the CLIPPER™ sequencer. The remainder of the sequencing reaction can be stored at -20°C for subsequent use.

The CLIPPER™ sequencer is set-up as described in the ***OPENGENE Automated DNA Sequencing System User Manual***. Run parameters for the CLIPPER™ sequencer are 54°C/ 1300volts/ 0.5sec sampling/35min run/50% laser power. The samples loaded included 2 ul each of the forward and reverse sequencing reaction products for the target gene, differentially labeled, for example with CY5.0 and CY5.5 cyanine dye labels. Once the run is completed, the base-called data is analysed by comparison of the test sequence to the rpoB sequence database in GENELIBRARIAN™ . This sequence alignment compares the test sequence to the standard control sequence and allows sequence ambiguities to be assessed. Once edited the test sequence can be screened for antibiotic resistance-associated mutations using GENELIBRARIAN™.

Testing for multiple types of antibiotic-resistance mutations can be carried out using a hierarchical assay, as summarized in Fig. 2. At present molecular biological methods for the detection of *M. tuberculosis* are only performed on AFB smear-positive sputum samples. These methods serve as confirmatory tests for the presence of *M. tuberculosis*. In addition to these molecular biological methods, the culture-based procedures for *M. tuberculosis* detection (BacTec liquid culture, agar plate and slant cultures) are performed in parallel. AFB smear-negative sputum samples are processed with only the culture-based detection procedures (Figure 1).

In the present invention both AFB smear-positive and smear-negative sputum samples can be processed using both culture-based and molecular biological methods. A limitation of the AFB stain methodology is it's limit of detection. If a sputum sample has a mycobacterial concentration of less than 5000 bacteria/ul the AFB stain will be negative. In addition to this is the observation that the decontamination procedure used to prepare the sputum sample usually kills 10-20% of the mycobacteria present. This would suggest that two-thirds of the AFB smear-negative samples potentially contain mycobacteria. In practice 10-20% of the AFB smear-negative samples are culture-positive for *M. tuberculosis* (Ontario Public Health Laboratory). This level of mycobacteria is easily detected by molecular biological methods and is therefore incorporated in the present invention.

The hierarchy proposed incorporates tests that specifically detect M. tuberculosis (rpoB), detect mutations in genes associated with resistance to the "first-line" antibiotics used to treat *M. tuberculosis* infections (rpoB, katG, rpsL/s12, PR, embB, pncA)

and detect other species of mycobacteria (23S) in the absence of M. tuberculosis (Figure 2). Group I analyses are performed before both Group II and Group III. Group I analysis will provide information on the antibiotic resistance status to rifampin (rpoB), isoniazid (katG), streptomycin (rpsL/s12) and azithromycin (23S). In addition the rpoB amplification indicates the presence of *M. tuberculosis* and in the absence of rpoB amplification the 23S sequence allows identification of most of the clinically relevant mycobacterial species. Group II analysis provides information on antibiotic resistance mutations in the "second-line" antibiotics used to treat *M. tuberculosis* infections namely, isoniazid (PR), ethambutol (embB), pyrazinamide (pncA) and ciprofloxacin (gyrA). Group III contains gene targets in which mutations associated with antibiotic resistance are infrequently found. This protocol permits specific gene targets to be examined according to the local treatment procedures since the both antibiotics used to treat *M. tuberculosis* infections, and thus the associated antibiotic resistance mutation patterns, vary geographically. As shown in Figure 2 the culture-based methods are performed in parallel. The molecular biological methods would permit the identification of M. tuberculosis from both AFB smear-positive and smear-negative sputum samples and further provide information on the antibiotic resistance profile of these samples well in advance of current culture-based methods. This information would be crucial to the initiation of appropriate and effective antibiotic treatment regimens for *M. tuberculosis* infections.

Examples

A pool of DNA samples from antibiotic-sensitive *M. tuberculosis* isolates was obtained from the LCDC, Health and Welfare Canada. Ottawa, Ontario. Wild-type sequence traces, for all gene targets known to harbor mutations in antibiotic-resistant *M. tuberculosis*, were generated.

A panel of DNA samples from five phenotypic streptomycin-resistant *M. tuberculosis* isolates was obtained from the Public Health Laboratory, Ontario Ministry of Health, Toronto, Ontario. These DNA samples were examined for antibiotic resistance-associated mutations in all 10 antibiotic gene targets listed above. Streptomycin resistance-associated mutations were detected in the rpsL/s12 gene in four isolates. Parallel antibiotic resistance-associated mutations in the rpoB (rifampin), katG (isoniazid), PR (isoniazid),

embB (ethambutol), pncA (pyrazinamide) and gyrA (ciprofloxacin) genes were also identified which underscores the importance of examining all the gene targets for first-line antibiotics used in the treatment of *M. tuberculosis*. A summary of the results is shown in Table 1.

The following references are cited herein and are incorporated herein by reference for all states which allow such incorporation.

a DL Williams et al. (1994). Characterisation of rifampin resistance in pathogenic mycobacteria. Antimicrob Agents Chemother 38: 2380-2386.

b WH Haas et al. (1997). Molecular analysis of katG gene mutations in strains of Mycobacterium tuberculosis complex from Africa. Antimicrob Agents Chemother 41: 1601-1603.

c S Sreevatsan et al. (1997). Analysis of the oxyR-ahpC region in isoniazid-resistant and -susceptible Mycobacterium tuberculosis complex organisms recovered from diseased humans and animals in diverse localities. Antimicrob Agents Chemother 41: 600-606.

d A Telenti et al. (1994). Genotypic assessment of isoniazid and rifampin resistance in Mycobacterium tuberculosis: a blind study at the reference laboratory level. Antimicrob Agents Chemother 35: 719-723.

e C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of Mycobacterium tuberculosis in Japan. J Appl Microbiol 83: 634-640.

f C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of Mycobacterium tuberculosis in Japan. J Appl Microbiol 83: 634-640.

g MA Lety et al. (1997). A single point mutation in the embB gene is responsible for resistance to ethambutol in Mycobacterium smegmatis. Antimicrob Agents Chemother 41: 2629-2633.

h A Scorpio et al. (1997). Characaterisation of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis. Antimicrob Agents Chemother 41: 540-543.

i C Xu et al. (1996). Fluoroquinilone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant Mycobacterium tuberculosis. J Infect Disease 174: 1127-1130.

j KA Nash et al. (1995). Genetic basis of macrolide resistance in Mycobacterium avium isolated from patients with disseminated disease. Antimicrob Agents Chemother 39: 2625-2630.

Table 1

gene (antibiotic)	OPH#1 bp/codon/aa	OPH#2 bp/codon/aa	OPH#3 bp/codon/aa	OPH#4 bp/codon/aa	OPH#11 bp/codon/aa
rpoB (rifampin)	cac526tac, His526Tyr	tcg553ttg, Ser553Leu	cac526gac, His526Asp	tcg553ttg, Ser553Leu	wt
katG.1 (isoniazid)	agc513acc, Ser513Thr	agc513acc, Ser513Thr	agc513acc, Ser513Thr	wt	wt
oxyR-ahpC PR (isoniazid)	g541a	wt	wt	wt	g541a
fabG (isoniazid)	wt	wt	wt	wt	wt
rpsL/s12 (streptomycin)	wt	aag43agg, Lys43Arg	aag43agg, Lys43Arg	aag88agg, Lys88Arg	aag43agg, Lys43Arg
16s/rrs (streptomycin)	wt	wt	wt	wt	wt
embB (ethambutol)	wt	gtc292ttc, val292phe	wt	wt	wt
pncA (pyrazinamide)	tcc65tct, Ser65Ser	wt	att133aat, Ile133Asn	wt	tcc65tct, Ser65Ser
gyrA (ciprofloxacin)	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr
23s (azithromycin)	wt	wt	wt	wt	wt

CLAIMS

1. A method for detection and characterization of *Mycobacterium tuberculosis* present in a sample, comprising the steps of:

(a) obtaining a sputum sample suspected of containing *M. tuberculosis*,

(b) performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the rpoB, katG, rpsL/s12 and 23S genes for the presence of antibiotic-resistance inducing mutations; and

(c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic-resistance inducing mutations.

2. The method of claim 1, wherein the second sequencing procedure evaluates PR, embB pncA and gyrA genes for the presence of antibiotic-resistance mutations.

3. The method of claim 3, further comprising the step of performing a third sequencing procedure when *M. tuberculosis* was detected in step (b) to evaluate 16S/rrs and mabA genes for the presence of antibiotic-resistance mutations.

4. The method of any of claims 1 to 3, wherein the first sequencing procedure for rpoB is performed using amplification primers as set forth in Seq. ID Nos. 1 and 2 and sequencing primers as set forth in Seq. ID. Nos. 3 and 4.

5. The method of any of claims 1 to 4, wherein the first sequencing procedure for katG is performed using amplification primers as set forth in Seq. ID Nos. 6 and 7 and sequencing primers as set forth in Seq. ID. Nos. 8 and 9.

6. The method of any of claims 1 to 5, wherein the first sequencing procedure for rpsL/s12 is performed using amplification primers as set forth in Seq. ID Nos. 21 and 22 and sequencing primers as set forth in Seq. ID. Nos. 23 and 24.

7. The method of any of claims 1 to 6, wherein the second sequencing procedure for 23S is performed using amplification primers as set forth in Seq. ID Nos. 46 and 47 and sequencing primers as set forth in Seq. ID. Nos. 48 and 49.

8. The method of any of claims 1 to 7, wherein the second sequencing procedure for PR is performed using amplification primers as set forth in Seq. ID Nos. 11 and 12 and sequencing primers as set forth in Seq. ID. Nos. 13 and 14.

9. The method of any of claims 1 to 8, wherein the second sequencing procedure for pncA is performed using amplification primers as set forth in Seq. ID Nos. 36 and 37 and sequencing primers as set forth in Seq. ID. Nos. 38 and 39.

10. The method of any of claims 1 to 9, wherein the second sequencing procedure for embB is performed using amplification primers as set forth in Seq. ID Nos. 31 and 32 and sequencing primers as set forth in Seq. ID. Nos. 33 and 34.

11. The method of any of claims 1 to 10, wherein the second sequencing procedure for gyrA is performed using amplification primers as set forth in Seq. ID Nos. 41 and 42 and sequencing primers as set forth in Seq. ID. Nos. 43 and 44.

12. The method of any of claims 2 to 11, wherein the third sequencing procedure for 16S/rrs is performed using amplification primers as set forth in Seq. ID Nos. 26 and 27 and sequencing primers as set forth in Seq. ID. Nos. 28 and 29.

13. The method of any of claims 2 to 12, wherein the third sequencing procedure for mabA is performed using amplification primers as set forth in Seq. ID Nos. 16 and 17 and sequencing primers as set forth in Seq. ID. Nos. 18 and 19.

14. A kit for evaluation of antibiotic-resistance mutations in a sample of *Mycobacterium tuberculosis*, comprising one or more pairs of amplification primers and one or more matched pairs of sequencing primers for amplification and sequencing regions within

the genome of M. tuberculosis, characterized in that the amplification and sequencing primer pairs are selected from among:

(a) amplification primers of Seq. ID Nos. 1 and 2 in combination and sequencing primers of Seq. ID Nos. 3 and 4;

(b) amplification primers of Seq. ID Nos. 6 and 7 in combination and sequencing primers of Seq. ID Nos. 8 and 9;

(c) amplification primers of Seq. ID Nos. 11 and 12 in combination and sequencing primers of Seq. ID Nos. 13 and 14;

(d) amplification primers of Seq. ID Nos. 16 and 17 in combination and sequencing primers of Seq. ID Nos. 18 and 19;

(e) amplification primers of Seq. ID Nos. 21 and 22 in combination and sequencing primers of Seq. ID Nos. 23 and 24;

(f) amplification primers of Seq. ID Nos. 26 and 27 in combination and sequencing primers of Seq. ID Nos. 28 and 29;

(g) amplification primers of Seq. ID Nos. 31 and 32 in combination and sequencing primers of Seq. ID Nos. 33 and 34;

(h) amplification primers of Seq. ID Nos. 36 and 37 in combination and sequencing primers of Seq. ID Nos. 38 and 39;

(i) amplification primers of Seq. ID Nos. 41 and 42 in combination and sequencing primers of Seq. ID Nos. 43 and 44; and

(j) amplification primers of Seq. ID Nos. 46 and 47 in combination and sequencing primers of Seq. ID Nos. 48 and 49.

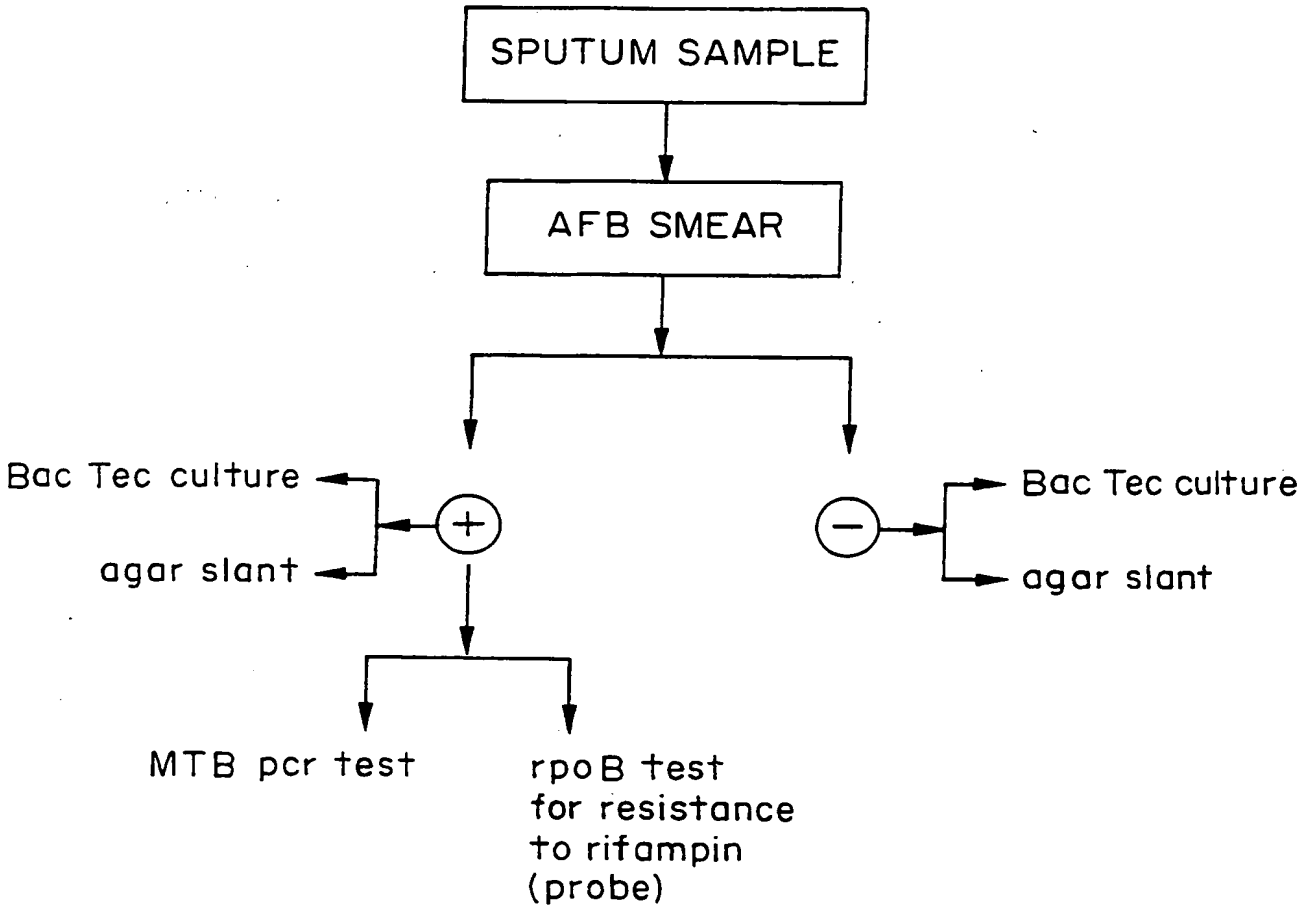


FIG. 1

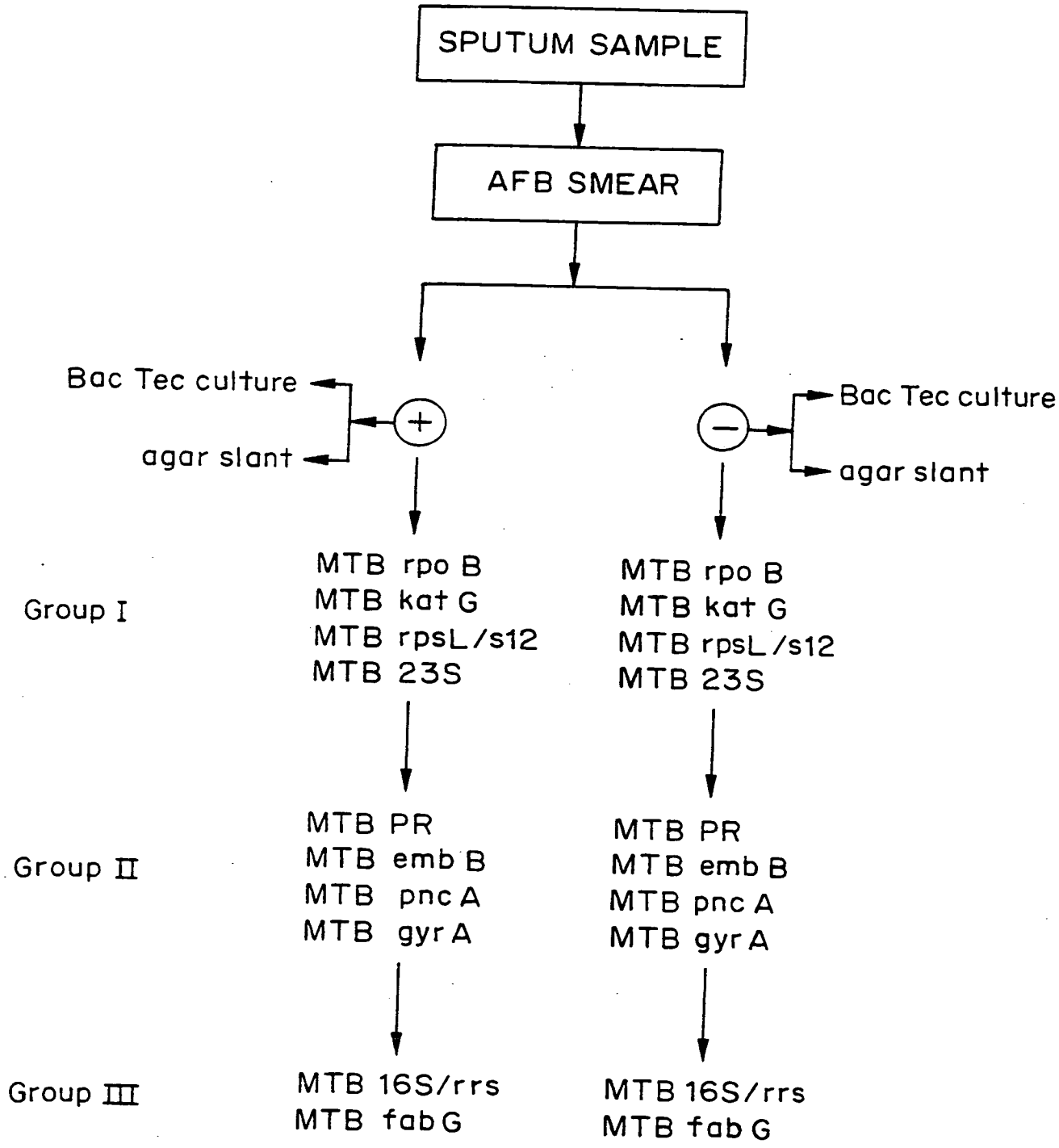


FIG. 2

SEQUENCE LISTING

<110> Visible Genetics Inc.
Shipman, Robert

<120> Method and Kit for the Characterization of Antibiotic-Resistance Mutations in Mycobacterium tuberculosis

<130> VGEN.P-055-WO

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<150> 60/111,794
<151> 1998-12-11

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cgcgaagccc ggccacggcc ggctagcacc tcttggcggc gatgccgata aatatggtgt 180
gatatatcac ctttgcctga cagcgacttc acggcacgat ggaatgtcgc aaccaaatgc 240
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cgcctaccag ctcaccgctc tcatcggcgg tgacctgtcc aaggtcgacg ccaagcagcc 360
cggcgactac ttcaccacta tcaccagtga cgaacaccca ggcaagtggc gggtggtgtt 420

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<400> 16
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<210> 17
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<212> DNA
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<210> 18
<211> 20
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<220>
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<400> 18
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<210> 19
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<210> 20
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agcgtaaccc cagtgcgaaa gttcccgccg gaaatcgcag ccacgttacg ctcgtggaca 180
taccgatttc ggcccggccg cggcgagacg ataggttgtc ggggtgactg ccacagccac 240
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<210> 22
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<210> 23
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<223> s12-5s sequencing primer

<400> 23
cggtagatgc caaccatcca 20

<210> 24
<211> 20
<212> DNA
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<400> 24
gcatcagccc ttctccttct 20

<210> 25
<211> 420
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> rpsL/s12 (streptomycin resistance)

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gcctatcagc ttgttggtgg ggtgacg 147

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<400> 31
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<210> 33
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<220>
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cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgctggtt 180
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<210> 39
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<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-3s sequencing primer

<400> 39
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<210> 40
<211> 561
<212> DNA
<213> Mycobacterium tuberculosis

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acaccggact attcctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgcggac 240
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<400> 43
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<220>
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<210> 50
<211> 300
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<220>
<223> 23S (macrolide/azithromycin resistance)

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cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccggga 180
ccttcactac aacttggtat tggtgttcgg tacggtttgt gtaggatagg tgggagactt 240
tgaagcacag acgccagttt gtgtggagtc gttgttgaaa taccactctg atcgtattgg 300

INTERNATIONAL SEARCH REPORT

Int .tional Application No
PCT/CA 99/01177

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAPUR V ET AL: "Application of automated DNA sequence analysis for mycobacterium species identification and detection of mutations associated with antibiotic resistance in Mycobacterium tuberculosis" ABSTRACTS OF THE INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 34, 1994, page 163 XP000901974 see abstract D71 ---	1-3
X	SUZUKI Y ET AL: "Detection of kanamycin-resistant Mycobacterium tuberculosis by identifying mutations in the 16SrRNA gene" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 36, no. 5, May 1998 (1998-05), pages 1220-5, XP000901934 the whole document --- -/--	1-3

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 April 2000	28/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

Int. ational Application No
PCT/CA 99/01177

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No
Y	HONORE N ET AL: "Streptomycin resistance in mycobacteria" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 38, no. 2, February 1994 (1994-02), pages 238-42, XP000901931 page 239, paragraph 2 ---	1-3
Y	SCORPIO A ET AL: "Characterization of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 41, no. 3, March 1997 (1997-03), pages 540-543, XP000901990 page 540 -page 542, paragraph 4 ---	1-3
Y	ALANGADEN GJ ET AL: "Mechanism of resistance to amikacin and kanamycin in Mycobacterium tuberculosis" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 42, no. 5, May 1998 (1998-05), pages 1295-97, XP000901991 the whole document ---	1-3
Y	HEYM B ET AL: "IMPLICATIONS OF MULTIDRUG RESISTANCE FOR THE FUTURE OF SHORT-COURSE CHEMOTHERAPY OF TUBERCULOSIS:A MOLUCULAR STUDY" LANCET THE,GB,LANCET LIMITED. LONDON, vol. 344, no. 8918, 30 July 1994 (1994-07-30), pages 293-298, XP002039609 ISSN: 0140-6736 the whole document ---	1-3
Y	WO 97 23650 A (DUNN JAMES M ;LEUSHNER JAMES (CA); STEVENS JOHN K (CA); VISIBLE GE) 3 July 1997 (1997-07-03) page 10, paragraph 1 -page 11, paragraph 4; example 7 ---	1-3
A	WO 95 33074 A (MAYO FOUNDATION ;HOFFMANN LA ROCHE (US)) 7 December 1995 (1995-12-07) page 3, paragraph 3 -page 6, paragraph 3 ---	1-3
A	WO 95 33851 A (INNOGENETICS NV ;BEENHOUWER HANS DE (BE); PORTAELS FRANCOISE (BE);) 14 December 1995 (1995-12-14) claims 2,22 ---	1-3

-/--

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INTERNATIONAL SEARCH REPORT

Int. .tional Application No
PCT/CA 99/01177

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No
P,X	NUESCA D ET AL: "RAPID DETECTION OF ANTIBIOTIC RESISTANCE-ASSOCIATED MUTATIONS IN 10 GENE TARGETS IN MYCOBACTERIUM TUBERCULOSIS USING THE OPENGENE(R) SYSTEM " ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 99, 30 May 1999 (1999-05-30) - 3 June 1999 (1999-06-03), page 636 XP000891874 see abstract U-13 -----	1-3

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir. tional Application No
PCT/CA 99/01177

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		EP 0870059 A	14-10-1998
		WO 9741257 A	06-11-1997
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		CZ 9603612 A	16-07-1997
		EP 0771360 A	07-05-1997
		JP 10500857 T	27-01-1998